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# ON THE DESTRUCTION OF COMPLEMENT BY HEAT.\*

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WILFRED H. MANWARING.

From the Pathological Laboratory of Indiana University.

[Work aided by the Rockefeller Institute for Medical Research.]

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In work with specific cytotoxic sera, it is often necessary to eliminate the action of the complement, or thermo-labile substance. To do this, it is customary to heat the serum from 55° C. to 60° C. for from 30 to 60 minutes.

During the course of experiments on the thermogenesis of auxilysins, it was necessary to determine, with some degree of accuracy, the exact time at which the complement is destroyed, when serum is so heated. In one such experiment, a flask of 350 c.c. of normal serum was immersed in a thermostatic water-bath at 59° C. Samples of the serum were removed, at two-minute intervals, and tested for the presence or absence of complement. With much surprise it was noted that the complement was apparently completely destroyed, at the end of eleven minutes, in spite of the fact that by that time the contents of the flask had reached a temperature of but 53° C.

This gave a very different conception of the lability of complement from the conception gained from the routine methods of complement destruction. Experiments were, therefore, undertaken to determine, somewhat accurately, the thermal-destruction point of this substance.

To do this, 1 c.c. of complement-bearing serum was

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\* Reprinted from the Transactions of the Chicago Pathological Society, vol. vi, pp. 425-427.

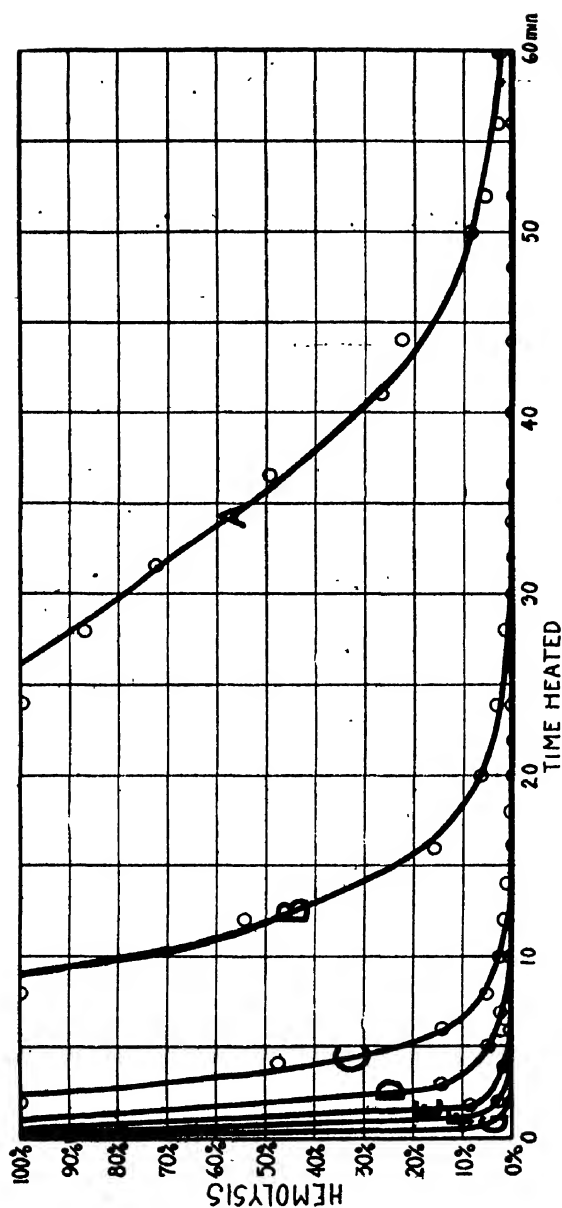


Fig. 1.—The Destruction of Complement by Heat. Curves show loss of reactivating power as normal goat serum is heated at different temperatures for different periods of time. The experiment was so planned that no apparent diminution of hemolytic power would take place till over 80 per cent. of the complement had been destroyed. A = curve when serum is heated to 49° C.; B = curve at 51° C.; C, at 53° C.; D, at 55° C.; E, at 57° C.; F, at 59° C.; and G, at 61° C. Curves made with same corpuscles, same sera, and on the same day.

placed in each of a dozen or more small test-tubes. The test-tubes were supported, about an inch apart, in a thermostatic water-bath, and, at stated intervals, removed and cooled in ice water. The serum in each tube was then tested for complement.

In making the test, there was added to each tube an amount of amboceptor large enough to give 100 per cent. hemolysis with about 20 per cent. of the complement originally present in the tube. No diminution in hemolytic power, therefore, would be discernible till over 80 per cent. of the complement had been destroyed. After this a further decrease in complement would be made evident by lessened hemolysis.

The results of such an experiment are shown graphically in Figure 1. From this it is seen that, when the serum is heated to 49° C. (Curve A), diminution in complement becomes evident in about thirty minutes, but that traces of complement remain undestroyed at the end of an hour. When heated to 51° C. (Curve B), diminution is evident in ten minutes and apparent complete destruction takes place in thirty-five minutes. At 53° C. (Curve C), complete destruction is shown in fourteen minutes; at 55° C. (Curve D), in twelve minutes; at 57° C. (Curve E), in eight minutes; at 59° C. (Curve F), in four minutes, and at 61° C. (Curve G), in two minutes.

It is thought that a more careful study of this phenomenon may throw light on the molecular composition of complement.

#### SUMMARY.

Normal goat serum, heated to 61° C., for two minutes, completely loses its power to reactivate a hemolytic goat serum made inactive by heat. Similar destruction of complement takes place at 59° C. in four minutes; at 57° C., in eight minutes; at 55° C., in twelve minutes; at 53° C., in fourteen minutes, and at 51° C., in thirty-five minutes. At 49° C., complete destruction has not yet taken place in sixty minutes.

Read April 9, 1906.

*Reprinted from Transactions of the Chicago Pathological Society,  
June 11, 1906.*

# ON THE PRODUCTION OF AUXILYTIC<sup>1</sup> AND ANTILYTIC SUBSTANCES IN HEATED SERUM.<sup>2\*</sup>

From the Pathological Laboratory of the Indiana University.

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WILFRED H. MANWARING.

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About a year ago, in work done in the University of Chicago,<sup>3</sup> it became evident that in order to understand the quantitative laws governing the action of hemolytic serum it was necessary to take into account, not only the amboceptor and complement usually regarded as the active components, but a third serum component as well. This third component is the substance present in normal serum after the complement has been destroyed by heat. To this component the name "complementoid" is currently applied, under the assumption that it is a degeneration product of complement. The evidence, however, indicates that this component is not a single substance, but a mixture of a large number of different substances, none of which have yet been proved experimentally to be in any way related to complement. The component will, therefore, be referred to in this paper simply as the *third serum component*.

Last November, I had the pleasure of presenting to the Society certain preliminary experiments<sup>4</sup> that show the action of this third component to be a very complex one. If increasing amounts of the third component are added to a constant amount of hemolytic serum, there is obtained, in certain experiments, a gradual increase in hemolytic power. Such an action is shown graphically in Curve A, Figure 1. In other experiments there is a very rapid increase in this power, followed by its gradual

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\* Reprinted from the Transactions of the Chicago Pathological Society, vol. vi, pp. 427-437.

1. *Adjuvant*, to increase.

2. Work aided by the Rockefeller Institute for Medical Research.

3. See Journal of Infectious Diseases, vol. 11, 1905, p. 460.

4. See Transactions of this Society, vol. vi, p. 319.

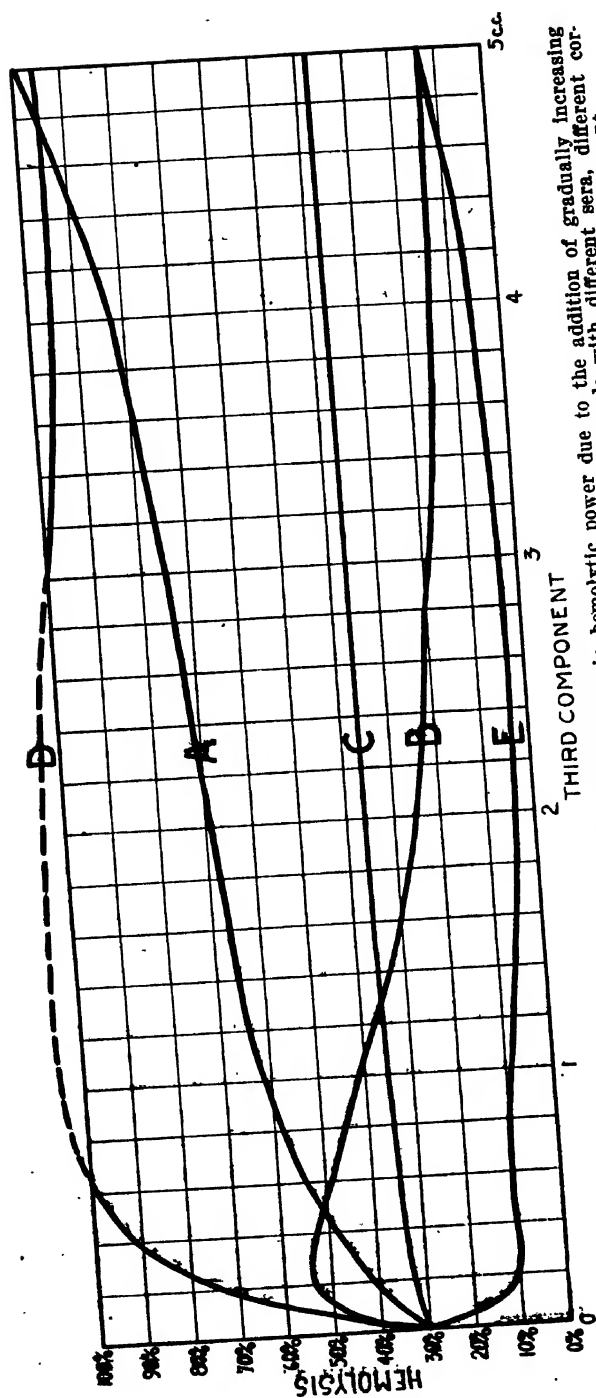


Fig. 1.—Third Component Curves. The curves show the changes in hemolytic power due to the addition of gradually increasing amounts of third component, to a constant amount of hemolytic serum. The curves were made with different sera, different cor-  
puscles and on different days. They are given here to show the remarkable variability in the action of the third component.

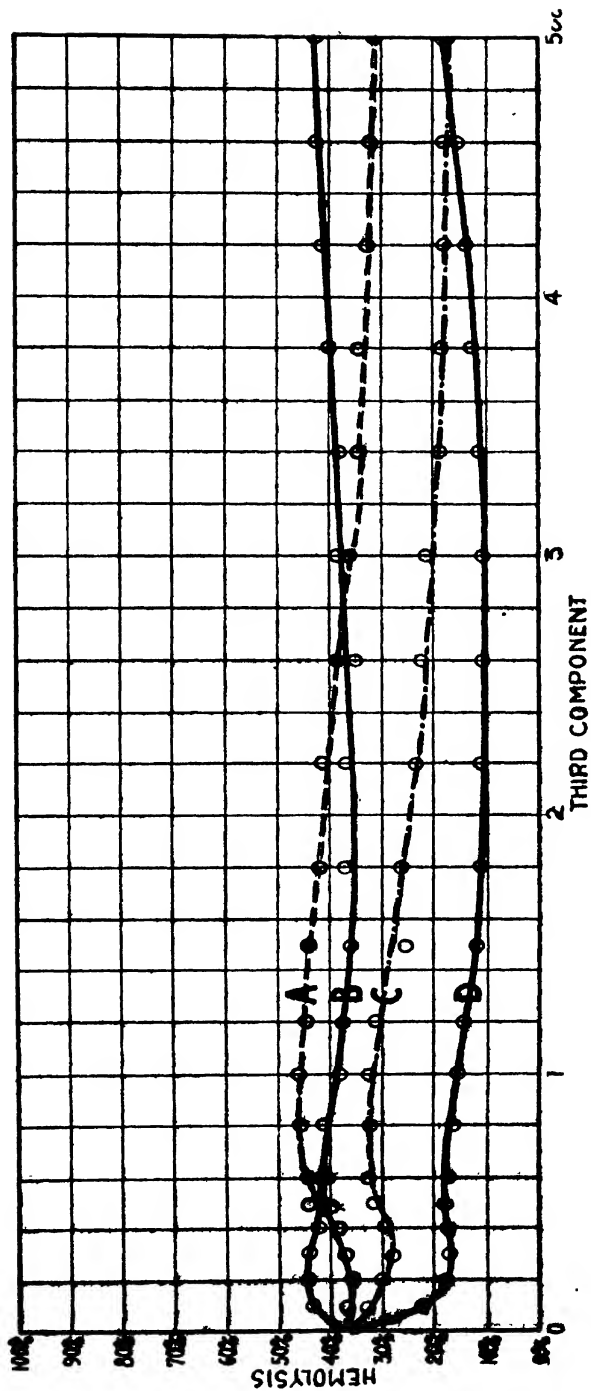


Fig. 2.—Comparison of Third Components of Different Normal Sera. Curves show effect on hemolytic power of adding increasing amounts of four different normal sera, heated to 56 C. for 60 minutes, to a constant amount of hemolytic serum. Curves made with the same hemolytic serum, the same corpuscles, and on the same day.



decrease (B, Fig. 1), while in rare cases the third component is practically without effect (C, Fig. 1).

Since November, curves have been obtained showing a purely decreasing action of the third component on hemolysis (E, Fig. 1), and other curves showing a remarkable increasing action (D, Fig. 1). Work was undertaken to determine the cause of this remarkable variability.

It is evident that there may be two causes for it. First, it is conceivable that the serum of different normal animals may differ markedly in this component. And, second, it is conceivable that differences may be produced in the same serum by differences in the way the serum is treated in different experiments. Work was undertaken to test both these possibilities.

In order to compare the third components of different sera, blood was drawn from several normal animals on the same afternoon and the sera allowed to separate in the same ice-chest. The next forenoon, 50 c.c. of each serum was measured out into flasks of the same size and heated to 56° C., for 60 minutes, in the same thermostatic water-bath, and under conditions that assured perfect uniformity of heating. Third component curves were plotted with the resulting sera.

Four curves, obtained in this way, are shown in Figure 2. A second experiment, performed in the same way, but in which the sera were heated to 59° C. for 60 minutes, gave a similar series of curves, four of which are shown in Figure 3. The curves in these two figures show that the sera of different animals, treated under identical conditions, possess widely different third components.

This result is not only of broad biologic significance, but is of special importance in serum pathology. It shows the impossibility of the exact quantitative analysis of sera, till a method is devised by which the action of the third component can either be eliminated, or, at least, made uniform in different sera. Two sera, having exactly the same amboceptor and complement, may have widely different hemolytic powers, if the third component of one is antihemolytic, while that of the other is auxihemolytic.

To determine the effect of differences in mode of treatment on the nature of the third component, serum was drawn in large quantity from one animal, and equal

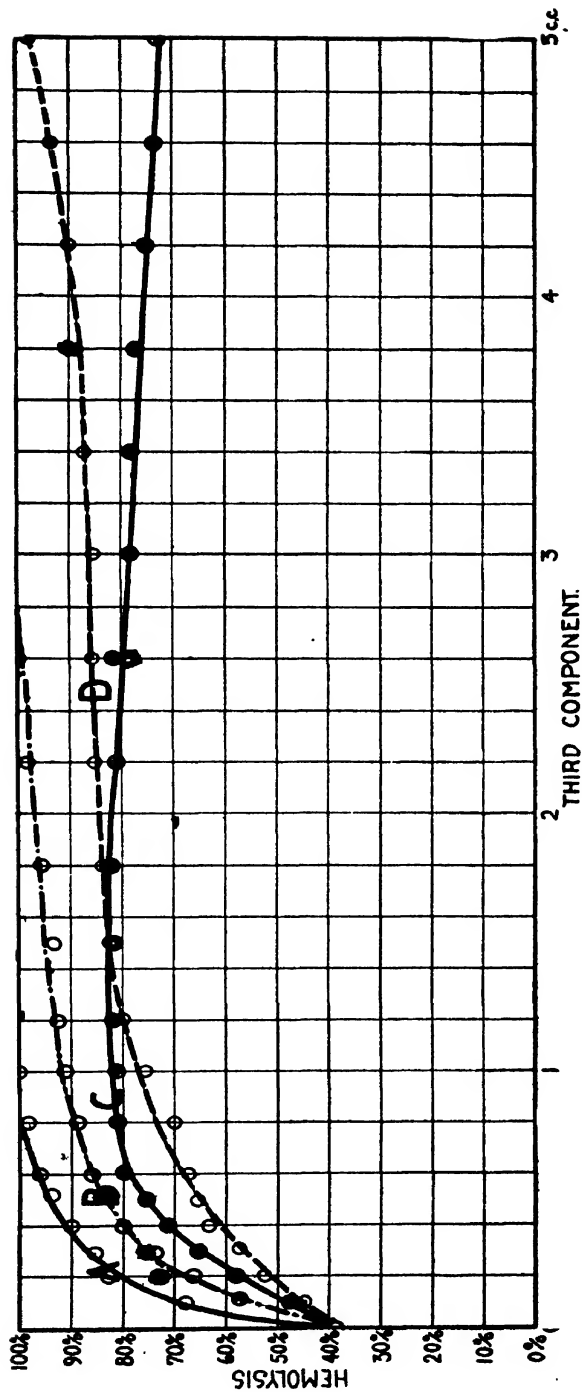


Fig. 3.—Comparison of Third Components of Different Normal Sera. Curves, as in Fig. 2, showing effects on hemolytic power of adding increasing amounts of four different normal sera, heated to 59° C. for 60 minutes, to a constant amount of hemolytic serum. Curves made with the same hemolytic serum, the same corpuscles and on the same day.

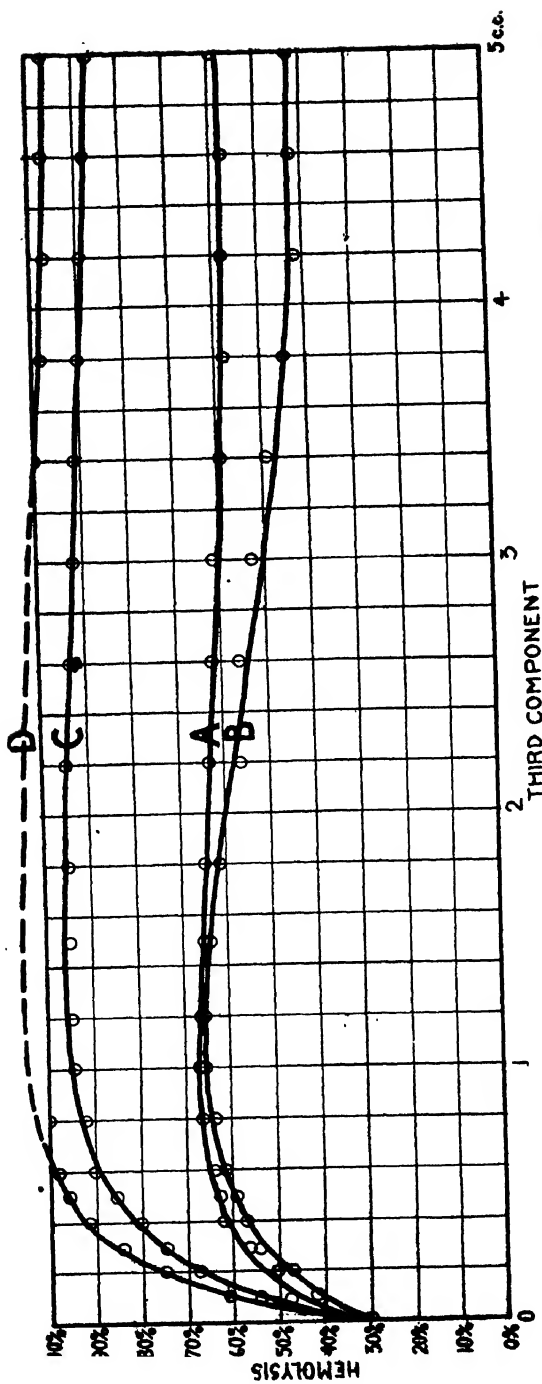


Fig. 4.—Effect of Time of Heating on the Third Component. Curves show changes in third component as the serum is heated for different periods of time. A = curve with serum heated to 56° C. for 30 minutes; B = curve with same serum heated to 56° C. for 60 minutes; C = curve, heated 100 minutes; and D, 160 minutes. Curves made with same sera, same corpuscles, and on the same day.

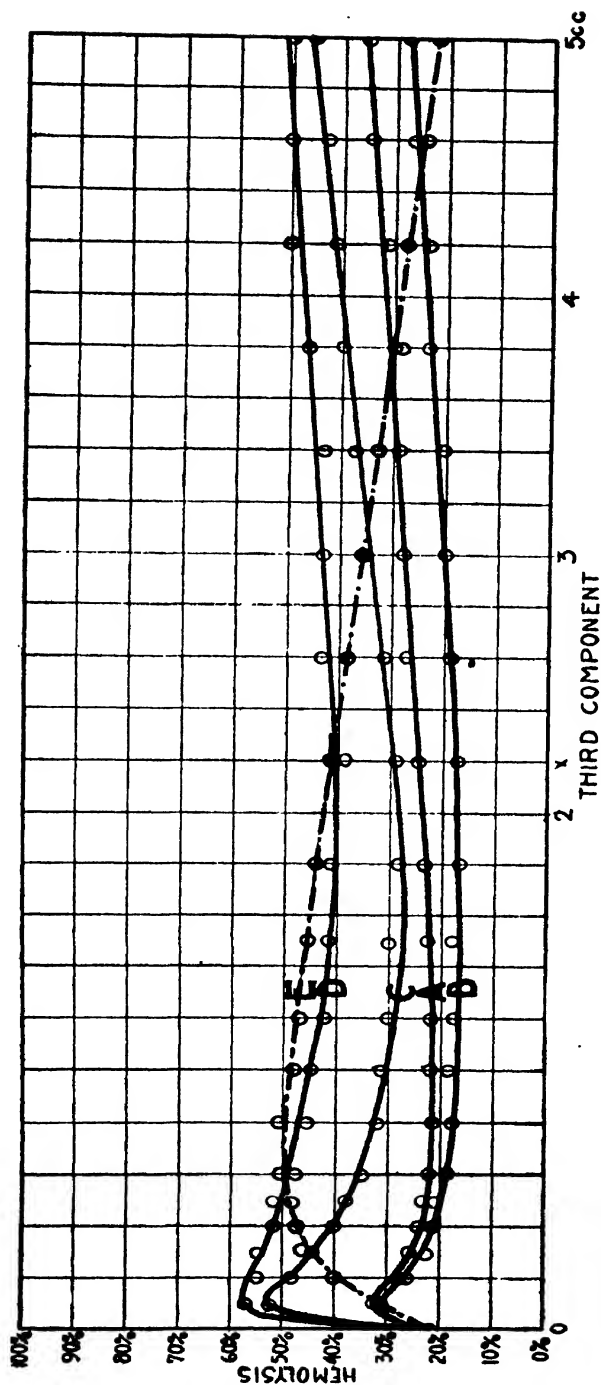


Fig. 5.—Effect of Time of Heating on the Third Component. Curves, as in Figure 4, showing changes in third component as the serum is heated for different periods of time. A = curve with serum heated to 56° C. for 35 minutes; B = curve with same serum heated to 56° C. for 65 minutes; C = curve, when heated 165 minutes; D, 240 minutes; and E 330 minutes. Curves made with the same sera, the same corpuscles and on the same day. The curves differ from those in Fig. 4, in that, in place of a constant amount of hemolytic serum, a constant amount of an artificial hemolytic amboceptor-complement mixture was used.

volumes of this were heated in the same water-bath for different lengths of time. Third component curves were then plotted.

Four curves, obtained in this way, are shown in Figure 4 and five similar curves in Figure 5. These curves show that differences in the mode of treatment of the same serum may produce widely different third components.

A hasty examination of these curves shows that one of the most constant effects of prolonged heating is the production of auxilytic substances (see Curve D, Fig. 4). There is also evidence of the production of antilytic substances (Curve B, Fig. 5) and of secondary changes in the nature of the auxilysin (Curve E, Fig. 5). Experiments were planned to follow the successive changes in auxilytic and antilytic power, as the serum is heated for different periods of time.

To do this, a flask of serum was heated in a thermostatic water-bath and accurately measured samples of this serum removed at five, ten or twenty minute intervals and immediately cooled in ice water. A constant amount of hemolytic serum was then added to each sample and the hemolytic power of the resulting mixtures tested.

A graphic representation of the result, obtained with serum heated to 56° C., is shown in Figure 6. This shows that, following the complete destruction of complement (A), there is a gradual production of an antilytic substance, which reaches its maximum in 60 minutes (B). Following this, there is a quite rapid production of a primary auxilysin, which attains a maximum in about two and a half hours. The primary auxilysin is then, in part at least, destroyed (D), after which there is a gradual production of a secondary auxilysin. This apparently has not reached its maximum at the end of ten hours' heating (E).

A similar curve, obtained by heating a second serum to 59° C., is shown in Figure 7. This shows the same general phenomenon, except that here the events occur with greater rapidity. The production of the antilytic substance in this experiment is apparently masked by earlier and more marked production of the primary auxilysin.

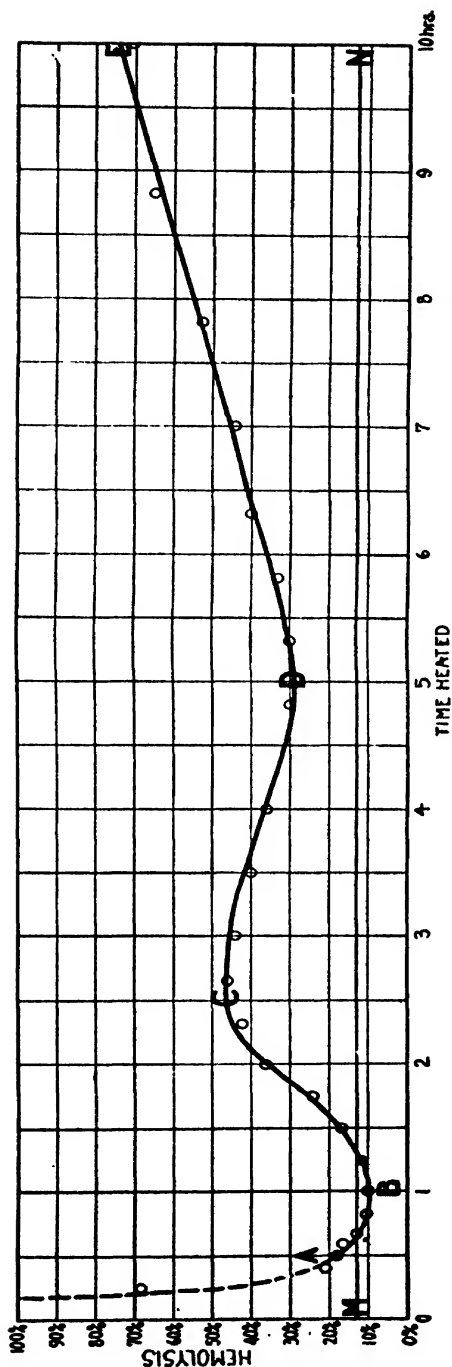


Fig. 6.—Thermo-Genesis of Auxilysin. Curve A-E shows the successive changes in auxilysin and antilytic power of the same amount of normal serum when heated to 56° C. for ten hours. The constant amount of hemolytic serum used in the experiment, was in itself capable of producing 14 per cent. hemolysis (M-N). Dotted portion of A-E shows curve before the complete destruction of complement. Curve A-E shows four phenomena: (i) the production of a small amount of antihemolysin, after the serum has been heated for 60 minutes; (ii) the comparatively rapid production of a primary auxihemolysin as the serum is heated beyond that period of time, the auxilysin reaching its maximum in about two-and-a-half hours; (iii) the partial destruction of the primary auxilysin as the serum is heated longer than two-and-a-half hours; and (iv) the production of a secondary auxihemolysin, the secondary auxilysin apparently not having reached its maximum at the end of ten hours.

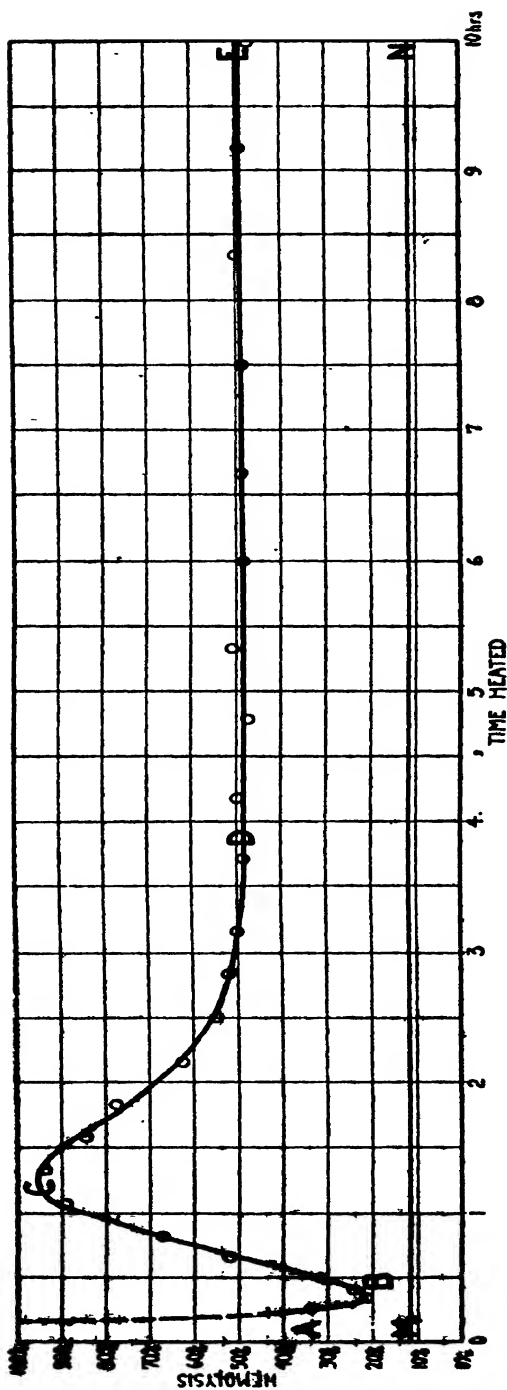


Fig. 7.—Thermo-Genesis of Auxilysin. Curve, as in Fig. 6, showing changes in auxilysin power when the serum is heated to 53° C. Curve shows the same general phenomenon as Figure 6, except that the events occur with much greater rapidity. The production of the antilysin (B) is apparently masked by the earlier and more marked production of the primary auxilysin.

## SUMMARY.

1. After the destruction of the complement by heat, there is left, in normal goat serum, a third component, which in different experiments shows auxilytic, antilytic or negative properties, when tested with goat serum immunized against sheep corpuscles.

2. The third components obtained from different normal goats, under identical circumstances, differ widely in their effect on hemolytic action.

3. The third component in the same serum differs widely under different experimental conditions.

4. Heating normal serum to  $56^{\circ}$  C., there is produced at first a weak antilytic substance, which is soon succeeded by a comparatively strong primary auxilysin. The primary auxilysin is, in part at least, destroyed and is succeeded by a secondary auxilysin. This apparently has not reached its maximum at the end of ten hours' heating.

5. Heating the serum to  $59^{\circ}$  C., produces approximately the same effects, except that the events succeed each other with greater rapidity.

Read April 9, 1906.





STUDIES UPON CALCAREOUS DEGENERATION.

By OSKAR KLOTZ.

## STUDIES UPON CALCAREOUS DEGENERATION.\*

### V.—THE RELATION OF EXPERIMENTAL ARTERIAL DISEASE IN ANIMALS TO ARTERIOSCLEROSIS IN MAN.

By OSKAR KLOTZ,  
*Resident Pathologist, Royal Victoria Hospital, Montreal.*

#### PLATE XXIV.

Since Josué first produced arterial changes in rabbits by means of adrenalin there has been a whole host of experimenters who have repeated and confirmed his work. Although Josué was the first to produce arterial lesions by means of adrenalin, he was preceded by Gilbert and Lion who were successful in bringing about arterial changes, by means of bacteria and their toxins; these lesions they believed simulated arteriosclerosis in man. Sumikawa, too, caused inflammatory changes near the arteries in rabbits, and believed that the vascular changes were identical with those found in arteriosclerosis. However, there is no doubt that arterial changes have been produced with most success by the inoculation of adrenalin. Erb, Baylad and Albarède, Külbs, Fischer, Scheidemandel, and Lissauer have experimented with adrenalin, and have noted the vascular changes. In the main their observations agree, but details of minor importance have been emphasized by some of them. B. Fischer has further found that digitalin when inoculated intravenously produces lesions similar to those caused by adrenalin.

The French workers, Josué and Baylad and Albarède, are most emphatic in maintaining that the intimal changes found in the vessels of experimental animals are of prime importance. The others, with whom I agree, hold that the medial lesions are the principal changes caused by adrenalin. The intimal changes,

\* This study was carried out at McGill University, Montreal, under a grant from the Rockefeller Institute for Medical Research.

which are rarely found, are of a secondary nature, or else are brought about by a process quite distinct from that producing the medial changes.

On account of the importance of a proper interpretation of these experimental lesions, and of the relation which they bear to the diseases of the arteries in man, I feel justified in making a histological comparison of the experimental and human lesions. It is impossible to call the macroscopical changes in the rabbit's aorta atheroma or arteriosclerosis unless the histological examination supports this view. I do not doubt that some have been misled in the use of these terms by the naked-eye appearance of the intima, while others have used the term arteriosclerosis in its broadest sense. Should the latter course be adopted, it should be recognized that experimental arteriosclerosis in rabbits is entirely distinct from sclerosis of the aorta in man. Moreover, as I shall point out later, the term atheroma should not be used in connection with the adrenalin lesions.

It is unnecessary for me to give in detail the methods of experimenting on animals, further than to state that my methods resemble those of previous investigators and more particularly those described by B. Fischer. The pure solution of adrenalin chloride, one in a thousand, was injected into the ear veins of rabbits.

Microscopical lesions, such as dilatation of the blood-vessels, hæmorrhages in the brain, and aneurismal distentions of the larger vessels, were noted. The most frequent lesions were situated in the descending arch of the aorta, where white calcified plaques formed the chief naked-eye alterations. The aneurisms were found to be situated in the centres of these white plaques which have been described by some as atheromatous areas. Histological examination shows that these plaques, at an early stage, are situated entirely in the media and that the calcification which occurs here is the secondary result of a degenerative, chiefly fatty, change which takes place in the media. Calcification is never found in the intima, and only rarely are there any intimal changes. The intimal changes which occur are of two kinds: either there is a

local heaping up of endothelial cells at a point where no degenerative changes of any kind have taken place, or there is thickening of the intima underneath the endothelial cells, chiefly due to proliferation of the connective tissue, in which slight fatty changes may occur. Such being the case, the term atheroma must be discarded in describing the experimental arterial lesions in rabbits.

As I have pointed out elsewhere, the primary change in the media, preceding the stage of calcification, is fatty degeneration of the muscle cells, followed by fatty degeneration of the elastic fibres. In the earliest lesions, before any macroscopic change can be noted in the vessels, fine granules of fat are aggregated about the nuclei of the muscle fibres. These granules of fat have an arrangement not unlike that seen in fatty degeneration of the heart muscle. From this stage of degeneration the process advances rapidly so that in a few days the median zone of the middle tunic of the vessels is in an advanced stage of degeneration. Fischer has already noted the rapidity with which the muscle fibres change under the influence of adrenalin inoculation, and hence has applied the name arterionecrosis. Since the degenerative changes are not so rapid as to prevent the appearance of fatty degeneration, and since there proceeds with the apparent necrosis a process of calcification, the name proposed by Fischer is a little misleading. I must grant that sections stained with hæmatoxylin and eosin give a picture of an active necrosis, but with Weigert's stain alone, or combined with Sudan III, the necrotic appearance disappears.

My studies lead me to accept the histological descriptions given by Fischer and others, except, as I have stated, that I find fatty changes in the muscle and elastic fibres preceding the deposit of the calcium salts. The mode in which these calcium salts are laid down has also been discussed in full in another paper, but as regards the sites of the calcium deposit I wish to speak more fully.

In sections stained with hæmatoxylin and eosin, it is seen that the lesion lies in the middle zone of the media and that in the early stages the muscle fibres become granular and lose their nuclei. With this degeneration of the muscle fibres, which must

necessarily be accompanied by softening of the tissue, the elastic fibres are packed more closely together, leaving less room between them for the muscle cells. At these points, where there is a loss of the muscle elements, the vessel wall is distinctly weakened, and partially gives way, leading to small aneurismal sacculations. In advanced lesions these aneurismal dilatations are the most marked macroscopic changes that are found.

When the muscle cells in the media have undergone fatty degeneration, the condition advances, until the cells become fairly loaded with the fat granules. Nuclear degenerations then become apparent and soon the cells die, leaving only their outline marked by the deposit of the fat granules which were in them. These fat granules do not coalesce but remain as isolated particles. Passing through the stage of fatty acids and soaps<sup>1</sup> they are converted into calcium compounds of the fatty acids, and hence the calcium deposit occurs as a fine sandlike material distributed in the sites of the former fatty granules. Following closely upon this change, the elastic fibres also become fatty, as Jores has observed, and go through the same process of calcification as the muscle cells. There is now a fine granular deposit of calcium at the site of the muscle fibres and rigid zig-zag calcified elastic fibres lying between them. This picture is not seen in the advanced stages of the calcification of aorta in rabbits, for the calcium salts have coalesced more or less to form larger masses, so that the former histological structure of the media is entirely lost. In no instance of experimentally calcified vessels in animals has primary calcification of the intima been noted. Occasionally I have seen very advanced experimental arterial disease in which the calcification of the media extended immediately below the intima.

If we are to accept Jores's definition of arteriosclerosis, namely, that the disease consists of a hyperplasia with degeneration of the musculo-elastic layer of the intima as seen in the human aorta, these experimental lesions would not fall in this category. I am not, however, inclined to accept this restricted

<sup>1</sup> See the studies upon calcareous degeneration by the writer. *Jour. of Exper. Med.*, 1905, vii, 633.

definition of arteriosclerosis, particularly as regards the human disease.

Lissauer compares the experimental lesions in arteries of rabbits to syphilitic arterial disease in man, pointing out that syphilitic arteritis attacks particularly the media. He falls short of a proper comparison. The main factor in the experimental lesions is the calcification of the muscle and elastic fibres in the media, and this never takes place in syphilitic disease of the vessels. In syphilitic arteritis the main reaction, consisting of an inflammatory change with new formed blood-vessels, an infiltration of small cells, and later the production of connective tissue, takes place in the outer third of the media. The blood vessels affected by adrenalin, on the contrary, show no reparative reaction in the media, the process being entirely a degenerative one. If a reaction does occur, it is secondary to a medial degeneration, and this reaction consists of a heaping up of the endothelial cells in the intima.

I would point out that although the lesion does lie in the media—and especially in the middle zone of the media—there are no changes in the vasa vasorum. All the hypotheses, maintaining that the action of adrenalin on the vasa in the vessel wall is the prime factor in the production of the necrosis, are based upon general inferences regarding the action of this drug in other parts; the histological appearances give no support to this theory.

• A second arterial disease which Lissauer wishes to compare with the vascular changes caused by adrenalin is the so-called neurotic angiosclerosis. He points out that Lewaschew and also Fraenkel found that by injuring or cutting the nerve supply of vessels, they became infiltrated with connective tissue, and that the change lay only in the media. Their results are quite contrary to those of Jores, who found that neither injury to a nerve nor the complete severance of it had any effect on the histological structure of the vessel it supplied. In syphilitic arterial disease, as I have mentioned, and in the neurotic lesion, calcification of the media is never seen.

There is, however, an arterial disease in man which simulates the

experimental arterial degenerations in every respect. This disease I have spoken of elsewhere as Moenkeberg's type of arteriosclerosis. This disease, as Moenkeberg and others have pointed out, affects the vessels of the extremities—the vessels of the muscular type—that is, arteries in which the muscle tissue predominates in the media. From this lesion the clinician makes the diagnosis of arteriosclerosis when he finds the radials hardened like “pipe stems” or “beaded like a trachea.” It is now definitely known that this form of arterial disease is distinguishable from arteriosclerosis as we find it in the human aorta. Moenkeberg's type of arteriosclerosis affects the media alone, causing degenerative changes. The intima is affected neither by degenerative nor by reparative changes, or if such changes are present they are secondary to or parts of another disease and not associated with changes taking place in the media. This disease of the media affects the vessels of the extremities—the femorals, tibials, internal and external iliacs, radials, and brachials being particularly susceptible.

Preceding the deposition of calcium salts in the media, there are degenerative changes which consist chiefly of fatty degeneration, which is visible to the naked eye, and can be distinctly traced in the media when the vessel is cut open longitudinally. This early fatty degeneration in the media differs from that of arteriosclerosis of the aorta in that the fatty plaques do not stand above the surface of the intima, but can be seen through this membrane. One is convinced by the microscopical picture, that in the early stages the muscle cells undergo a fatty degeneration characterized by the deposition of the finest fat globules. The calcium salts, too, are found to be deposited as fine sandlike granules in the degenerated muscle fibres. In the early stages of degeneration, calcium granules are found in double wedge-shaped aggregations lying between the elastic fibres, and this grade of calcification is not to be recognized by the naked eye. However, with the constant accumulations of fresh calcium deposit, the granules lie very close together and then coalesce. At this stage, when the calcium granules are becoming densely aggregated, we find that the vessel wall decreases in thickness; there is a thinning of



the media with a bulging outward of the wall at this point. Distinct multiple aneurismal dilatations are of common occurrence in these vessels, so that the outer contour of the vessel is quite irregular. From the inner surface of the vessel it is seen that the calcified areas form depressions which are lined by smooth and unchanged intima. Microscopically, the elastic fibres are seen to be stretched into straight lamellæ and no longer show the wavy character found in healthy vessels. The elastic fibres, too, are calcified and are lying more closely together, although in advanced cases this is difficult to distinguish, as the calcium deposit in the muscle and elastic fibres have run together into a solid mass. In consequence of the constant tension and stretching of the elastic fibres, they have become calcified in the position that they had in these vessels, and thus we find the straight calcified laminae. Where calcification of elastic fibres has advanced beyond the general area of calcareous degeneration, and, too, where contractile muscle fibres have persisted between them, these elastic fibres have taken on a zig-zag outline due to fractures.

Microscopically one can demonstrate that the running together of the calcium granules takes place in the middle of the media, and that at either end of such a mass are outrunners of calcified elastic fibres and granular degenerated muscle fibres. The internal elastic lamina is stretched as it passes over the calcified and aneurismal portion of the media.

The intima occasionally shows some reaction over the areas of medial degeneration, the endothelial cells being heaped up. This intimal change is, however, entirely secondary and has nothing to do with the primary cause of the medial change, and further is not a necessary accompaniment of the medial degeneration.

Moenkeberg holds that the adventitial changes are in great part confined to thickening of the intima of the vasa vasorum, but points out that this limitation is not a constant feature in the disease. There are some who claim that the degeneration of the media is due to occlusion of the vasa vasorum causing poor nutrition of the media. However, the changes in the vasa vasorum are too inconstant to allow us to draw any definite con-

clusion, and, moreover, it is very hard to estimate the amount of change in these small arterioles with no definite wall, especially when they are collapsed and empty.

Pure medial calcification is commonly found as an accompaniment of advanced age, occurring most frequently after the age of fifty. But, as Marchand has pointed out, it occurs also in young individuals. The disease affects males more frequently than females, the ratio being about six to one. It is further of interest that medial calcification occurs in the vessels of those extremities which are most active; thus in right-handed individuals it is found more pronounced on the right side, while in those whose duties keep them constantly on their feet the femorals are most affected. Again, the disease is particularly common among the laboring classes.

When the experimental lesion in animals and the medial arteriosclerosis in man are compared, they present great similarity in their anatomical, histological, and, it may be, their etiological characters. In each case the essential lesions are confined to the media, the changes in the intima being of a secondary nature or else the result of a different disease. In both lesions the process in the media rapidly leads to calcification of the muscular and elastic elements, fatty change preceding the deposition of the calcium salts. Aneurismal dilatations at the sites of the calcium deposits in the media often occur with both lesions, and in the dilated part the vessel wall is much thinned as the result of the loss of muscular elements and the packing together of the elastic fibres.

Both diseases are brought about or accompanied by an increased blood pressure; in the one it is artificially produced by the use of adrenalin and involves the general circulation, while in the other it is localized in parts, the extremities, where increased work, straining and constriction of the muscles, lead to heightened pressure. Whether this heightened blood pressure acts directly on the vessel walls to cause the medial degeneration, or whether by the stretching of the arteries the vasa vasorum are in part or wholly pinched off, leading to focal necrosis in the media, is not wholly clear. Certainly from the focal character

of the experimental lesions and from the annular arrangement of the degeneration in the vessels of the extremities in man, I should feel very much inclined to think that the vasa played an important rôle in the production of these changes. However, we must not lose sight of the fact that in the experimental animals the toxic character of the substances inoculated may be an important factor in the production of the lesions, as Fischer has pointed out.

Both of these lesions must be distinguished from the intimal form of arteriosclerosis as found in the aorta, and the presence of one condition does not indicate the presence of the other.

Several clinicians have made the observation that when pure medial arteriosclerosis is present in the vessels of the extremities without any changes in the aorta there is no hypertrophy of the left heart, while, on the contrary, if the aorta is affected with arteriosclerotic changes the heart shows hypertrophy. This fact suggests that while the aorta is healthy and elastic, it relieves, to a great extent, the increased blood pressure which would naturally follow the hardening of the peripheral vessels, while, on the other hand, if the elasticity of the aorta is destroyed, the increased pressure is directly transferred to the heart, leading to hypertrophy of the organ. The experimental hardening of the walls of the aorta apparently confirms this view, since hypertrophy of the heart frequently occurs with it.

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Fig. 1.

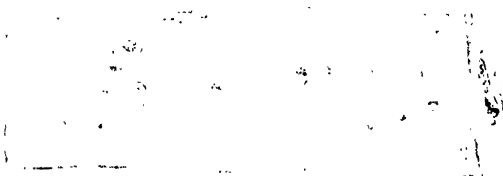


Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.



Pearce and Stanton.—*Trans. Assoc. of Amer. Phys.*, 1905, xx, 513.

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#### EXPLANATION OF PLATE XXIV.

Fig. 1.—Femoral artery (natural size), showing pittings and small aneurismal dilatations in the areas of calcification of the media.

Fig. 2.—Aorta of rabbit (enlarged x 2), after injections of adrenalin, showing small calcified plaques with depressions and aneurismal bulgings in their centres.

Fig. 3.—Femoral artery (low power, Leitz obj. 3, ocular No. 2), showing calcification of the media with little change in the intima other than splitting of the internal elastic lamina.

Fig. 4.—Aorta of rabbit after injections of adrenalin (low power), showing calcification in the media with no change in the intima. Vessel wall shows aneurismal dilatation.

Fig. 5.—Femoral artery (high power), showing densely calcified media, along with a less intensely calcified area in which the elastic and muscle fibres are in the early stages of calcification.





THE PATHOGENESIS OF EXPERIMENTAL COLITIS,  
AND THE RELATION OF COLITIS IN  
ANIMALS AND MAN.

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There is no longer any real doubt that in the group of dysentery bacilli we possess the essential causes of the dysenteric inflammations of the intestine in man. This general statement is justified notwithstanding the facts that in man a class of inflammations of the intestine is caused by pathogenic amoebæ, and an indefinite group of intestinal inflammations, due to no common cause, is occasionally met with. The morbid conditions produced, on the one hand, by pathogenic amoebæ, and, on the other, by irritating foreign bodies, the bacteria of food poisoning and the salts of heavy metals, are, taken together, not very infrequent; but as compared with those caused by the dysentery bacilli they are relatively inconsiderable.

The experimental studies of the past ten or twenty years have shown that a considerable number of diverse poisons produce, in animals, inflammations of the intestine. Some of these poisons are of known definite composition, as the salts of mercury and some other metals; others are of indefinite nature, so far as our present knowledge reaches, as, for example, the peculiar poison contained in putrefying fluids. A third class of poisons of somewhat more definite nature, capable of causing inflammations of the gut, are of vegetable origin: these are the toxic products of bacterial growths, ricin, abrin, etc. While the precise chemical nature of these toxic bodies is still unestablished, the poisons themselves are sufficiently defined to permit of their employment under uniform conditions of experiment. The present paper deals with the effects produced on rabbits by inoculation, chiefly, of a toxic product obtained from the Shiga dysentery

bacillus, and a study of the manner in which the peculiar effects are brought about. In the course of the investigation it was found necessary to resort to the use of some other poisons, *e.g.*, corrosive sublimate and ricin, in order to bring out certain facts which the use of dysentery toxin had failed to establish, or to establish, beyond doubt, certain deductions drawn from the experiments with the dysentery toxin. The results of this experimental study are presented here because of their bearing upon the pathogenesis of colitis in man.

Two main groups of dysentery bacilli are now recognized: the "Shiga" and the "Flexner" groups. The first agree with the bacillus first isolated by Shiga from cases of Japanese dysentery; the second isolated by one of us (Flexner) from cases of Philippine dysentery. The two groups possess in common the capacity to set up dysentery in man. No profound distinction in this power can be drawn between the bacilli. The disease which they cause appears in sporadic, endemic, and epidemic forms. In the case of young children it would appear, from our present knowledge, that the Flexner type of dysentery bacillus causes much more frequently than the Shiga type certain varieties of ileo-colitis. The cultural distinctions between the two groups are considerable: the Shiga type of bacilli has a restricted power to ferment the sugars, while the Flexner type has a rather wide capacity of fermentation. The two groups are also separated by their agglutination reactions: they are less widely separated in respect to their reactions to immune sera. On the other hand, they are sharply separated by their capacity to yield, in fluid cultures, soluble toxins. The Shiga type of bacillus readily produces a soluble toxin, the Flexner variety either not at all or in minimal quantities. Shiga bacilli grown on solid culture media, killed at 60° C. or with chloroform, still yield, when suspended in water or salt solution, a toxin which goes over into the fluid. The Shiga bacilli easily undergo autolysis through which the toxin is liberated. The Flexner bacilli do not yield by autolysis, any more than in fluid culture, a toxic body to a surrounding fluid medium.

As tested on certain small laboratory animals—young guinea-

pigs, for example—the two types of bacilli are about equally toxic. This toxicity does not depend, necessarily, upon multiplication of the bacilli, for the dead micro-organisms are toxic. Hence the Flexner type of bacillus must yield in the animal body an active toxic product. As tested on rabbits, however, the Shiga type of bacillus is more toxic than the Flexner type. The rabbit is, indeed, very sensitive to the action of the Shiga dysentery bacillus or its toxin. The easily demonstrable effects are exerted upon the central nervous system and upon the intestine. Two kinds of intestinal lesions occur either separately or combined. The more common lesions appear in the small intestine: the gut is dilated, the vessels are injected, the Peyer's patches swollen and congested, and a faintly greenish-yellow glutinous or serous fluid fills the lumen. Although occurring less frequently the lesions in the large intestine are much more striking. The part of the large gut affected is, almost exclusively, the dilated cæcum and appendix. Rarely is the colon involved. The lesions vary in intensity: the coats of the gut are greatly thickened by inflammatory oedema, in which case the mucosa is yellowish-white and thrown into deep folds and corrugations, or with the oedema more or less hæmorrhage may be associated. At another time the transverse folds of mucous membrane are affected chiefly; they are swollen, the edges are hæmorrhagic, and pseudo-membrane is scattered over the surface. Or, again, the transverse folds are greatly and the intervening mucosa is less affected, and patches of swollen and hæmorrhagic mucous membrane, covered with a false membrane, appear upon and between the folds. The hæmorrhage may extend into the serous coat. It is impossible, in a brief space, to describe all the variations of appearance of the affected mucous membrane. There is, however, in many cases striking similarity in the lesions, as regards location and general pathological appearances, with the early diphtheritic dysenteric lesions in man. The masses of lymphoid tissue at the ileo-cæcal valve and in the appendix are invariably swollen in the rabbits, showing the large intestinal lesions, and frequently they are beset with small hæmorrhages. The mesenteric lymphatic glands are always swollen, oedematous, and congested.

There are considerable variations of susceptibility to the dysentery toxin among the rabbits. A part only of the rabbits which succumb to the toxin develop the nervous and large intestinal lesions. A given lot of toxin will cause in certain rabbits the nervous and intestinal lesions, and in still other rabbits transitory illness from which they recover, or death without visible lesions. Neither does there appear to be a marked relation between dosage and lesions, or even death. Of a given toxin 0.01 cubic centimeter may be fatal to one series of rabbits while another series may survive 0.1 cubic centimeter of the same poison, the weights of the rabbits having been about equal. Larger doses usually kill, but the period of survival varies considerably. The symptoms which follow the inoculations are diarrhoea, and paralysis and convulsions. The diarrhoea is sometimes bloody. The paralysis begins in the upper extremities and extends, at times, to the lower extremities. It may be incomplete. The animals may survive for days after the extremities are paralyzed. They lie on the side with the head drawn back, and in opisthotonic position. The convulsions are irregularly intermittent, and may be excited by disturbance of the animal. Towards the end of life the paralysis of the extremities becomes more marked. In general appearance these animals resemble rabbits in the paralytic stage of rabies. Although rabbits may survive many days after the onset of the paralytic symptoms, which may themselves be several days in developing, yet in some instances the symptoms of paralysis may appear within twenty-four hours and the animals survive this appearance only a few hours. Speaking generally the larger doses of toxin—0.5 to 1 cubic centimeter—produce marked symptoms and cause death earlier than the smaller doses.

The dysentery toxin which we are considering was first obtained by Conradi, whose results were briefly mentioned by v. Drigalski<sup>1</sup> and later were fully described by himself.<sup>2</sup> The method employed by Conradi consisted of aseptic autolysis of the cultures. Of his preparations 0.1 cubic centimeter was fatal,

<sup>1</sup>V. Drigalski.—*Veröffentlichungen der Medizinalabtheilung des Königlich Preussischen Kriegsministeriums, Berlin, 1902, Abt. 2, 107.*

<sup>2</sup>Conradi.—*Ueber lösliche durch aseptische Autolyse erhaltene Giftstoffe von Ruhr- und Typhusbazillen. Deutsche med. Wochenschr., 1903, xxix, 26.*

the symptoms preceding death being diarrhoea, collapse, and paralysis. In four rabbits which survived longer than 48 hours Conradi noted the appearance of diphtheritic inflammation of the intestine with which ulceration was associated. Neisser and Shiga<sup>3</sup> confirmed Conradi's findings except that they noticed chiefly the lesions of the small intestine in the poisoned rabbits. The number of authors who have prepared and studied the action of the dysentery toxin is now considerable. Vaillard and Dopter<sup>4</sup> found that when the rabbits succumbed to small doses in from 18 to 24 hours they exhibited the lesions of the small gut, and when larger doses were administered subcutaneously the lesions of the large intestine appeared. Rosenthal<sup>5</sup> and Todd<sup>6</sup> studied toxins obtained by growing the dysentery bacillus in bouillon for several weeks; up to six weeks the filtered cultures gave rise in rabbits to the intestinal and other lesions. Todd does not seem to have met with the inflammations of the large intestine. Kraus and Dörr<sup>7</sup> worked with filtrates of ten-day-old bouillon cultures and with saline extracts of fresh cultures of the dysentery bacilli. Both were toxic. In their opinion no autolysis is required since the living bacilli yield the toxin by extraction. They do not mention whether the characteristic symptoms and pathological changes occurred in their experiments. Lüdke<sup>8</sup> applied the method of Rowland and Macfadyean, namely grinding the bacilli under liquid air, which operation was followed by extraction with salt solution, to the preparation of a toxin. The product was fatal to rabbits, causing, however, chiefly the lesions of the small gut and only inconsiderable and rare lesions of the large intestine. Lüdke regards the poison as of the nature of endo-toxin, and he failed to obtain a true toxin by growing the bacilli of dysentery in various media. The exudate which is found in guinea-pigs after intraperitoneal injections of dysentery bacilli is, when free of all cells, still toxic. Kikuchi,<sup>9</sup> who described the symptoms produced by this substance, mentions paralysis but not lesions of the intestine.

There is no doubt that all who have studied the dysentery toxin were dealing with the same substance, and the differences in effect met with depend largely upon dosage and the variable re-

<sup>3</sup>Neisser und Shiga.—Ueber freie Receptoren von Typhus- und Dysenteriebazillen und über das Dysenterietoxin. *Deutsche med. Wochenschr.*, 1903, xxix, 61.

<sup>4</sup>Vaillard et Dopter.—La dysenterie épidémique. *Ann. de l'Inst. Pasteur*, 1903, xvii, 486.

<sup>5</sup>Rosenthal.—Das Dysenterietoxin (auf natürlichen Wege gewonnen). *Deutsche med. Wochenschr.*, 1904, xxx, 235.

<sup>6</sup>Todd.—On a Dysentery Toxin and Antitoxin. *Jour. of Hygiene*, 1904, iv, 480.

<sup>7</sup>Kraus und Dörr.—Ueber experimentelle Therapie der Dysenterie. *Wien. klin. Wochenschr.*, 1905, xviii, 1077.

<sup>8</sup>(a) Lüdke.—On the Dysentery Toxin. *Jour. of Path. and Bact.*, 1905, x, 328.

<sup>8</sup>(b) Lüdke.—Ueber die Gewinnung von Dysenterietoxin. *Berl. klin. Wochenschr.*, 1906, xliii, 3 and 54.

<sup>9</sup>Kikuchi.—Untersuchung über die Shiga-Kruseschen Dysenteriebazillus. *Archiv. f. Hyg.*, 1905, lii 378.

action of the rabbit to the injections. These animals succumb chiefly to the nervous poison (neurotoxin) which produces in the brain visible small hæmorrhages and in the spinal cord softening of the gray matter with and without visible hæmorrhages. The upper segment of the cord tends chiefly to be affected. The small intestinal catarrh is almost constantly met with, and the congestion of the blood-vessels beneath the serosa in the large bowel probably indicates that this portion of the intestine is also commonly affected by the slighter lesions. The discrepancies in the literature as regards the nervous and intestinal symptoms and lesions probably depend upon the different manner of their manifestations and the ease with which the former are detected. The absence of intestinal lesions in Kikuchi's experiments, does not depend upon a fundamental difference of action of the exudate and the autolysed poison, for in some of our experiments in which an exudate from the guinea-pig was employed, the intestinal lesions were obtained. Dysentery toxin is a stable substance as it retains its activity in sterile solution under toluol for many weeks, and resists the growth, as we found, of the common moulds. The slighter toxicity of old bouillon cultures would indicate that under certain conditions the toxin tends to depreciate in strength.

There is general agreement among those who have given special attention to the subject that the Flexner type of dysentery bacillus does not yield a soluble toxin. The only dissenting experience is that of Vaillard and Dopter<sup>10</sup> who claim to have obtained identical results with all types of the dysentery bacillus. Since it is not expressly stated that a mannite-fermenting dysentery bacillus was employed it is safe to assume that one of this group was not among the types studied by them.

For preparing the dysentery toxin we used a culture of the Shiga bacillus which had been passed through a series of guinea-pigs to maintain its virulence. The fatal dose for a guinea-pig of 200 to 250 grams was one oese, death taking place in less than twenty-four hours. The cultivations were made upon agar on large flat surfaces, upon which the bacilli grew for twenty-four hours. The growth was washed into sterile water or 0.85 per

<sup>10</sup> *Loc. cit.*, p. 488.



cent. salt solution, the organisms killed by exposure to 60° C. for one hour, and the suspension kept at 37° C. for varying periods. The toxic solution was obtained by filtration through a Berkefeld filter. The filtrate was preserved in the ice-box under toluol. The injections were made intravenously in rabbits.

Although aside from our main theme, which was to ascertain the manner in which the intestinal lesions in rabbits were produced, a series of experiments on the comparative toxicity of living, dead, and autolysed cultures was made. Our results do not uphold Kraus and Dörr who state that an active toxin can be obtained without autolysis, and they indicate, further, that the separated toxin is more effective than the same quantity of poison contained within the living or the dead bacilli.

TABLE I.

Series No.	Weight in grams	Amount injected	Nature of material injected	Results
26	2000	0.1 c.c.	living suspension	dead after 10 days
27	1620	"	"	lives
28	1920	"	suspension after 1 hr. at 60° C.	lives
29	1270	"	suspension after 1 hr. at 60° C.	dead after 5 days
30	1150	"	suspension autolysed 3 days	dead after 11 days
31	1150	"	suspension autolysed 3 days	dead after 2 days

TABLE II.

Series No.	Weight in grams	Amount injected	Autolysed — days	Results
23	1610	0.5 c.c.	2	lives
24	1570	"	3	dead after 2 days
25	1740	"	4	lives
32	2470	"	5	dead after 5 days
33	1270	"	6	" " 5 "
34	1300	"	7	" " 2 "
35	1300	"	8	" " 2 "
38	1700	"	9	lives
39	1310	"	10	dead after 3 days
66	1160	1.0 c.c.	15	" " 2 "
75	900	"	27	" " 6 "

Since Conradi believed that the toxin may be weakened by allowing the autolysis to continue longer than forty-eight hours, an experiment was made to determine this point. As Table II shows the toxin is still effective after a period of autolysis extending to twenty-seven days.

The preceding series of experiments serve also to confirm the statement already made, namely, that individual peculiarities play an important part in determining susceptibility to the poison. Of several rabbits the larger may succumb and the smaller escape, or those receiving the smaller doses may die and those a larger dose survive. But where very large doses, from two to five cubic centimeters, are injected, the animals much more rarely survive; and under these circumstances the animal receiving the largest dose usually succumbs the earliest.

The tendency of the diphtheritic lesions of the intestine to confine themselves to the cæcum and colon led early to the notion that retention of the fæces was the chief agent in causing the condition. All the earlier views are summed up in Virchow's paper which appeared in 1863 in which he defined his own belief that fæcal retention is a necessary condition to the production of diphtheritic dysentery. This view now dominates in the pathology of dysentery. It will be instructive to present, in abstract, Virchow's<sup>11</sup> argument in order that a comparison may be made of the conditions present in dysentery in man with those occurring in the experimental disease in the rabbits.

"Dysentery arises from the action of products of decomposition upon the intestinal mucosa already the seat of a superficial catarrhal inflammation. That this is true is shown most clearly by the manner of extension of the dysenteric process. The projecting points—those which come directly and remain longest in relation with the contents of the intestinal mucosa—are first and most severely affected. Rokitsansky is mistaken in saying that as a rule the dysenteric process extends from the valve to the rectum, taking on in intensity from above downwards. Generally speaking those portions of the large gut of which the contents remain longest in contact with the mucosa are most severely affected. Those parts are the cæcum, the hepatic, splenic, and sigmoid flexures, and the rectum. Should the intestine present abnormal flexures then the rule holds for them, should the normal flexures be accidentally wanting then this part of the

<sup>11</sup>*Virchow's Archiv*, 1863, v, 348.

gut remains relatively free of disease. In particular it is the projections of the mucous membrane, caused by the anatomical distribution of the muscle tissue, which are affected by preference. Hence the especial susceptibility of the mucosa along the insertion of the three longitudinal muscle bands, and of the transverse folds of the membrane. In that the diphtheritic process establishes itself first on the projecting and later on the intermediate mucosa, there arise those regular geographical figures which indicate the intensity and mode of spread of the lesion. When it is considered that the diphtheritic process does not begin regularly at a definite portion of the mucous membrane, as, for example, the rectum, but that this portion is often least involved while a higher level of intestine is often intensely affected, then the presumption is difficult to set aside that the pathological process is due to some agent which, acting locally, causes the lesions.

"Rokitansky (*Pathological Anatomy*, ii, p. 265) compares with right the anatomical changes in dysentery with those which caustic acids produce; and hence the question arises whether the necrosis is due to a direct chemical change in the tissues caused by an action starting from the surface. Dysentery begins, undoubtedly, as is the case with every superficial inflammation of the mucous membrane, as a simple catarrh, the lesions being met with only after the diphtheritic condition has appeared. Hence it is necessary to ascertain whether the increase from catarrh to diphtheria takes place without the action of local injurious agencies. It may in general be assumed that a simple intestinal catarrh, by reason of the presence of products of decomposition, is converted at those points where decomposition is going on into a diphtheria; and these products are contained chiefly in retained faecal masses."

In his paper on dysentery published in 1877 Virchow<sup>12</sup> states that diphtheria of the intestine may exist independently of dysentery and instances his findings in some cases of Asiatic cholera of diphtheritis of the intestines. What is of special interest, in this connection, is the statement that the diphtheritic process may involve the ileum, affecting partly the surface of Peyer's patches and partly the villi. "In the latter cases the mucosa was much thickened, firm, and disposed in high transverse folds, making it appear at first as if it were jejunal mucosa.

The increase in our knowledge since these writings has yielded the tangible agents of dysentery and set aside the notion of the action of the products of decomposition, unless, indeed, the toxic constituents of the dysentery bacilli be viewed as such products. Although we have secured this advantage of knowledge, no direct application of it to the pathogenesis of the lesions of dysentery has been attempted. It is clear from the

<sup>12</sup> *Charité Annalen*, 1877, ii, 706.

descriptions of the experimenters with dysentery toxin that the intestinal lesions in rabbits agree very closely with those of human dysentery. The small intestine is found constantly, or nearly so, in a state of catarrhal inflammation, and in a percentage of animals the large gut shows diphtheritis, œdema, and hæmorrhage.

None of the previous experimenters seems to have been impressed with the finer localization of the lesions in the large gut of the rabbit. Aside from the statement that the cæcum is the seat of predilection nothing is recorded. An analysis shows, however, that two main sets of lesions are encountered. In one the parts of the intestine chiefly affected are the transverse folds of the mucous membrane of which the tips or the entire fold may be involved. In some instances the intervening mucosa escapes, in others the pathological process appears there also, often as focalized areas, but sometimes as a more or less diffuse involvement. In the other a diffuse involvement of the cæcum appears to have been present from the start. The former cases present many points of similarity with the appearances described in human dysentery; while for the latter cases it may be said that in the Philippine Islands one of us (Flexner)<sup>13</sup> observed in autopsies upon American soldiers who died of dysentery, diffuse, hæmorrhagic lesions which agree essentially with the diffuse hæmorrhagic condition present in the rabbits.

The importance of this correspondence appears when it is considered that in man the noxious agent reaches the intestine directly through ingested materials, while in rabbits the poison is injected into the circulation and must reach the intestine through its blood supply. The conclusion, in the case of the rabbits, seems sufficiently obvious; the toxic material is secreted into the intestine and produces directly or indirectly pathological effects. But whether in man the noxious material acts directly or through the blood remains as before an open question.

The rabbit is not subject to infection with the dysentery bacillus when cultures are fed the animal; and we have found that

<sup>13</sup>On the Etiology of Tropical Dysentery, *Philadelphia Medical Journal*, 1900, vi, 414.

injection of cultures of the bacilli directly into the duodenum is apparently without effect in this animal. As was to be expected from this result the injection into the duodenum of considerable quantities (up to 10 c.c.) of the autolysed toxin is also without action. The rabbit is not, therefore, subject to poisoning with the dysentery bacillus or its toxic products when they are brought at once into the gastro-intestinal canal. All other forms of inoculation—subcutaneous, intraperitoneal, intravenous—are effective. It may therefore be assumed that direct contact of the bacilli or the toxin with the mucosa of the rabbit's gut cannot lead either to absorption unchanged or act injuriously upon the membrane. Since it was possible that the poison might be altered in the small intestine and reach the cæcum in an inactivated form, an experiment was carried out to ascertain whether direct contact of the toxin with the mucosa of the cæcum would lead to a different result. A rabbit was starved and given magnesium sulphate per os to empty the bowel. The peritoneum was opened and 1.0 cubic centimeter of dysentery toxin diluted with ten cubic centimeters of salt solution was injected directly into the cæcum and allowed to come into contact with the mucous membrane. No immediate effect was noted, and the animal showed, on being killed forty-eight hours later, no lesions of the intestine. The interpretation to be placed upon this experiment is clear, and all the experiments taken together would seem to indicate that the lesions of the gut present in the rabbits given the dysentery bacillus or its toxin, in such way as to bring the poisons into the blood, are caused not by direct effect upon the surface of the mucous membrane but indirectly through the act of elimination of the poison by the intestinal mucosa. In endeavoring to explain the absence of action of the toxin upon the intestine when given by mouth or injected into the lumen of the gut, tests were made on the resistance of the toxin to peptic and tryptic digestion, but no light was obtained. It was found that the toxin is destroyed by peptic digestion (artificial gastric juice) after twenty-four hours' contact, while 0.3 per cent. hydrochloric acid alone is without influence on the toxicity. On the other hand, the toxin

resists tryptic digestion for eighteen hours, but after a longer contact (at the end of three days) it is destroyed. The addition of solutions of erepsin, prepared by Cohnheim's method, to the trypsin does not accelerate the effects.

The toxin is moderately heat stable. Neisser and Shiga found that a temperature of  $75^{\circ}$  C. reduced greatly its action; Vaillard and Dopfer state that the temperature of  $70^{\circ}$  to  $75^{\circ}$  C. maintained for one hour is without effect, while at  $75^{\circ}$  to  $80^{\circ}$  C. the toxicity is diminished and at  $81^{\circ}$  C. destroyed. Rosenthal's specimen of toxin was weakened by heating to  $70^{\circ}$  C. Our results agree with those of Vaillard and Dopfer: the toxin studied by us was injured at  $80^{\circ}$  C. and destroyed at  $81^{\circ}$  C., those temperatures having been maintained for one hour.

It was noted in some earlier experiments by one of us (Flexner) that when rabbits are injected intravenously with the dysentery bacillus the living bacilli may be found in the bile. Notwithstanding the negative results obtained from direct injection of the bacilli and the toxin into the intestine, it was considered desirable, in view of the possibility of the intestinal lesions being eliminative in nature, to exclude not only the bacilli but also the entrance, possibly, of the toxin into the intestine with the bile. To accomplish this exclusion two methods were employed: the common bile duct was tied; and a biliary fistula was established. After ligating the duct in two places the portion lying between the ligatures was resected. A permanent biliary fistula was made by inserting a special cannula into the apex of the gall bladder and conducting the secretion through rubber tubing to the exterior. The common duct was always tied off. The operations were carefully conducted with all precautions and infection was readily avoided. Table III shows the somewhat surprising results of this series of experiments. While of the ten control animals five developed the characteristic lesions of the cæcum, of the eight animals in which the duct was ligated two only showed similar lesions, and of the ten animals in which a permanent fistula was established only one animal (Number 19) developed lesions of slight degree. As regards rabbit Number 35 it is safe to assume that the small hæmorrhage noted was caused by manipulation of the gut at the operation.

TABLE III.

Series No.	Weight in grams	Amount injected	Death after—days	Result as regards the le- sions of the cæcum
Controls				
81	1555	5mg. powdr.*	2	no lesions
83	1590	"	11	œdema and congestion, no hæmorrhages
5	1300	0.5c.c.toxin	5	marked œdema, no hæmorrhages
6	1630	0.5; 0.5; 2.0	lives	
8	2100	5.0	2	no lesions
9	2200	5.0	2	numerous hæmorrhages, with necroses
12	2150	2.0	1	no lesions
14	1570	2.0	2	no lesions
16	1540	0.5	4	very extensive hæmor- rhage and necrosis
20	2100	5.0	2	scattered hæmorrhages and necrosis
Tied duct				
82	1510	5mg. powdr.	9	no lesions
84	1605	"	9	œdema and hæmorrhages
1	1240	0.5; 1.0; 0.5	7	no lesions
2	1500	0.5; 1.0	3	no lesions
10	2140	5.0	3	no lesions
11	2140	2.0	1	no lesions
15	1520	2.0	4	œdema, no congestion, no hæmorrhages
17	1740	0.5	4	no lesions
Permanent biliary fistula				
3	1230	0.5	2	hyperæmia, no focal lesions
4	1530	0.5; 1.0	4	no lesions
13	1880	2.0	1	no lesions
18	1860	2.0	3	no lesions
19	2020	0.5	4	slight œdema, no hæmor- rhage.
21	2140	5.0	2	no lesions
27	2070	10.0	3 hrs.	no lesions
31	2500	5.0	1	no lesions
35	2100	1.0	1	area of hæmorrhage at pt. of cæcum nearest liver
51	2300	5.0	3	no lesions

In discussing these results it is perhaps desirable to point first to their apparent paradoxical nature. While the direct injection of dysentery toxin into the intestine is followed neither by death of the animal nor development of visible intestinal lesions, the exclusion of the toxin from the lumen of the gut by

\* Made by drying in thermostat at 37° C. the autolysed culture fluid.

cutting off the entrance of bile tends to prevent the lesions without, at the same time, preventing the fatal action of the poison. The nervous symptoms develop in animals in which the bile duct has been tied or a biliary fistula established. It would be wrong to conclude that the toxin exercised no influence on the body merely because the intestinal lesions were absent, for in a part of the fatal cases the striking lesions of the cæcum do not appear; and the less striking ones of the small bowel might well exist and be followed by recovery. The test of action is, indeed, a lethal result or the development of the nervous symptoms; for although some of the animals injected with small amounts of the toxin recover, yet none of those receiving into the circulation as large doses as may be introduced in the intestine without producing severe symptoms ever survive.

Hence one is brought to the conclusion that the poison which acts injuriously upon the intestine, through elimination, is also excreted by the bile. Indeed, it would agree best with the experimental results to conclude that of all the circulating poison at any one period a fraction only is eliminated by the intestinal wall, while the larger fraction passes out of the circulation through the liver. This larger fraction, entering the intestine with the bile, is then partially or wholly reabsorbed, only to be subjected in turn to the same series of events. According to this view the intestinal lesions are gradually developed by successive acts of excretion. It does not follow that this elimination takes place only in the cæcum, and that the absorption occurs only higher up in the gut. While it seems probable that the chief absorption does occur in the small intestine, the existence of lesions in this part of the intestine may point to it as being also, although in a minor degree, concerned with excretion, while there is nothing to prove that absorption may not also take place in the large intestine. No explanation is at hand for the failure of the toxin to be absorbed when introduced in mass into the small intestine. After this relation of the poisoning to the bile secretion into the intestine became apparent, we attempted, without success, to reproduce some of the symptoms



of the intoxication by mixing the toxin outside the body with rabbit bile and injecting the mixture into the small intestine.

When the bile is drained away a large part of the toxin is lost, but there still remains available, from the multiples of lethal doses injected, sufficient to cause death through the nervous injury. That the chief intestinal lesions should in these experiments be absent is not remarkable. It is surprising, however, to find that the lesions of the large gut occur more infrequently in the animals in which the bile duct is tied off than in animals not operated upon. It is true that the lesions of the small intestine have been present in some of these animals; and from this it has been concluded, first, that some excretion of the toxin occurs in the small intestine, and, second, that the liver holds back, from the blood, the accumulated toxin which it naturally would secrete into the bile. Whether the poison is merely stored in the liver or destroyed there has not been determined.

The experimental results stated, have brought us to the conclusion that dysentery toxin is both excreted by and absorbed into the blood from the intestine. In this respect the toxin is brought into conformity with certain organic (morphia) and inorganic (iron, manganese) substances which are both absorbed and excreted by the gastro-intestinal mucosa. But interesting as this fact taken by itself may be, our special object at present is to show, as far as possible, the extent to which this property of the toxin of being absorbed and excreted by the intestine may account better than the older views, emphasized by Virchow, for the development of the catarrhal and diphtheritic lesions of dysentery in human beings. The bacteriological studies of cases of dysentery in man examined post-mortem have shown the dysentery bacillus to be present throughout the intestinal tract. It is not limited to the large intestine, although it is, possibly, more abundant in the fibrinous exudations. All parts of the mucous membrane of the intestine can be made to yield the bacillus in culture irrespective of whether they are the seat of pathological changes or not. Since the development in any numbers of the dysentery bacillus does not take place

outside the intestinal tract, it must be concluded that the toxin, which causes the lesions and symptoms of the disease, is produced in the gut. It would agree well with the experimental results related to assume that in man the absorption of the dysentery toxin takes place in the small, and possibly also in the large intestine, but that, usually, the chief elimination is by way of the large intestine; and only under unusual conditions, as in the rare cases of diphtheritis of the small intestine, to any great extent by way of the small gut. This general statement of the manner of production of dysenteric lesions, the diphtheritic ones especially, in man does not exclude the possibility that certain secondary causes contribute to determine the extent of the lesions of the large intestine, among which retained faecal masses might play an important part.

On account of the variable action of the dysentery toxin upon the cæcum of rabbits, the need was felt of studying, by the methods employed in these experiments, more definite chemical substances which provoke eliminative lesions of the intestine. For this purpose corrosive sublimate and ricin were chosen. The lesions of the large intestine which follow the subcutaneous injection of corrosive sublimate in rabbits have been accurately described by Heilborn<sup>14</sup>, and those which result from ricin injections into the circulation by Ehrlich<sup>15</sup> and Flexner<sup>16</sup>. Mercury colitis is such a well-known condition that no special description of it is called for, but the fact may be mentioned that the small intestine escapes injury, while the cæcum and the first part of the colon are the seats of hæmorrhage, necrosis, diphtheritis, and ulceration. The most constant and most pronounced lesions are in the spiral folds of the cæcum. The striking lesions in ricin intoxication are general hyperæmia of the the small intestine associated with an excessive secretion of serum and mucous, and swelling of and punctiform hæmorrhages into the Peyer's patches. The lymphatic glands generally become swollen and hyperæmic, and numerous hæmorrhages

<sup>14</sup>*Arc. f. exp. Pathologie u. Pharmakologie*, 1878, viii, 361.

<sup>15</sup>*Deutsche med. Wochenschr.*, 1891, 976 and 1218.

<sup>16</sup>*Jour. Exp. Med.*, 1897, ii, 197.

occur in the omentum, mesentery, and serosa of the gut and abdominal wall.

TABLE IV.

Series No.	Weight in grams	Amount of HgCl <sub>2</sub>	Death after — days	Result as regards the lesions of the cæcum
Controls				
29	810	0.038	3	lesions of entire cæcum
39	1295	0.06	3	“ “ “
44	1550	0.03	1	no lesions
36	1030	0.03	1	“ “
57	1540	0.015	4	lesions of entire cæcum
60	1070	0.01	2	“ “ “
		0.015		
		0.01		
Tied duct				
48	1350	0.03	7	lesions confined to ileocæcal region
50	1290	0.03	1	2 small areas of necrosis
56	1310	0.015	1	lesions confined to ileocæcal region
Permanent biliary fistula				
49	1320	0.03	1	no lesions, except congestion
55	1810	0.015; 0.01	3	mod. lesions of entire cæcum, becoming more intense at end of organ toward the appendix
58		0.015; 0.01	2	slight lesions confined to ileo-cæcal region

In Table IV are given the results of injections of corrosive sublimate into three series of rabbits: (1) into a control series, (2) into a series in which the bile duct was tied and severed, and (3) into a series in which biliary fistula had been produced. Only in the animals with tied bile duct could any lesions be found when death took place within twenty-four hours after the first injection of the mercury. All the animals which lived two days or longer showed lesions, but in the case of the animal with a tied duct which succumbed after seven days, they were slight, while in the two animals with fistulæ, one dying on the second and the other on the third day, the lesions were much less severe than in the corresponding controls. It would therefore appear that mercury, as is the case with dysentery toxin, is reabsorbed after secretion by the bile into the intestine and excreted chiefly by

the cæcum and colon, which portion of the gut shows the severest grade of injury. As in the case of dysentery toxin, the process of elimination is a gradual one.

In order to test this view of the reabsorption of mercury by the bile from the small intestine enterostomy was performed on two rabbits. In the first the small intestine was attached to the abdominal wound at a point about two inches above the ileocæcal valve, after which the intestine was tied off above the valve. After closing the opening in the abdominal wall the small intestine was opened. There was free outward passage of contents. Two doses of sublimate of 0.02 gram each were given subcutaneously on October 17th and 18th. Rabbit dead on October 19th. Fistula opening patent; cæcum showed very few small lesions at the edges of the spiral valves; no lesions of the colon. In making the opening in the intestine of the second rabbit a small cannula was employed. October 16th, operation done; October 17th and 18th each 0.02 gram sublimate injected subcutaneously. Rabbit dead October 19th. The autopsy showed that the opening of the cannula was occluded by a mass of dried intestinal contents; so that no fluid could escape. The cæcum showed hæmorrhages into the spiral valve with superficial necrosis, and hæmorrhagic infiltration of the colon. This second experiment, through the accident of the closure of the cannula, becomes a convincing control for the first one. It may, therefore, be considered as rendered probable that the intestinal lesions in mercury poisoning are caused not necessarily by the mercury present originally in the blood, but by a process of successive elimination and reabsorption of the metal from the intestinal tract in which process the biliary secretion plays a highly important part.

In carrying out the experiments with ricin a sample made by Merck, the toxicity of which was about 0.1 milligram per kilo of rabbit, was employed. The control rabbit, weighing 1420 grams, was given 0.15 milligram of ricin intravenously on January 27th. Death occurred twenty-four hours later. At autopsy the characteristic lesions inclusive of increased fluid in the pericardium and peritoneum, hæmorrhages into the epicardium and omentum,

swelling and hæmorrhage of the superficial and deep lymphatic glands, much swelling of Peyer's patches, increase of mucoid fluid in the small intestine, and enlargement ( $\times 2$ ) of the spleen existed.

In a second rabbit, weighing 1470 grams, a permanent biliary fistula was made on January 26th, and immediately after the operation 0.15 milligram of ricin was injected. Death took place the next day. The autopsy showed slight swelling of and no hæmorrhage in the lymphatic glands, no excess of fluid in the pericardium and peritoneum, no hæmorrhages in the epicardium or peritoneum, slight swelling of Peyer's patches, and contracted (normal) condition of the small intestine. A repetition of this experiment, using twice the dose, was made. The control and operated rabbits were given 0.3 milligram per kilo of body weight. The former succumbed after nineteen, the latter after twenty-two hours. The lesions in the first were very pronounced and of a strikingly hæmorrhagic character. Considerable hæmorrhage had taken place into the mucosa and contents of the cæcum and appendix. In the fistulous rabbit the lesions were more marked than in the first operated animal and much less severe than in the control rabbit. While some of the peripheral lymphatic glands were not perceptibly enlarged, hæmorrhages had taken place into others, and still others were congested. The patches of Peyer were only slightly swollen; but a few small punctiform hæmorrhages occurred in them; the lymphatic tissue of the cæcum was swollen but free of hæmorrhages.

The experiments with ricin leave no doubt that the pathological effect of the poison is greatly modified by permitting the bile to escape from the intestine; and while the fatal outcome is, perhaps, only delayed by the operation, the profound difference in the tissue and vascular reactions indicate that an essential part or effective quantity of the poison leaves the body with the bile. But in addition the experiments prove that resorption of ricin takes place from the intestinal canal, and the lesions met with also point to an excretion by this organ.

The three sets of experiments which have been presented would seem to have important features in common. If it is

permissible to apply the chief facts of them to the interpretation of the production and localization of diphtheritic dysenteric lesions in man, it may be said that these lesions are due to the action of a toxin elaborated by the dysentery bacilli present in the diseased intestine, which toxin is first absorbed, in the main probably from the small intestine, and eliminated chiefly through the large intestine, which suffers injury through the act of excretion. In offering this view of the pathogenesis of dysentery we would add that the injury upon the tissues once inflicted in the manner mentioned, other micro-organisms than the dysentery bacillus doubtless come into action and complicate and increase the pathological effects of the dysentery toxin.

A study of the histology of the lesions of experimental colitis tends to confirm the conclusion that the poison which causes degeneration, necrosis, and inflammation of the caecum acts not first upon the surface of the mucous membrane, but upon the mucous membrane and the submucosa simultaneously. In the purely œdematous lesions the mucosa may entirely escape noticeable injury, even the surface epithelium being retained in normal appearance. It is, however, usual for the mucous membrane in these cases to show œdema also, especially of the layer next to the muscularis mucosæ. The exudate in these cases is an inflammatory œdema giving rise to fibrin formation in the submucosa; no striking number of polymorphonuclear leucocytes are seen in the tissues although in the veins they may be increased. The blood-vessels are usually congested.

When hæmorrhage is associated with the œdema the mucous membrane is much more altered. The extravasation of blood takes place chiefly in the mucous membrane and usually causes disintegration of the glandular tissue. Associated with the hæmorrhage is, often, necrosis of the cellular elements of the mucous membrane, the necrosis exhibiting itself by hyaline degeneration of the glandular cells, fragmentation, etc. In the cases in which there is much disintegration and necrosis of the mucous membrane, the submucosa shows, besides œdematous swelling, an invasion of polymorphonuclear cells, and thrombosis of lymph-vessels and of the dilated veins. There is no sharp line

between this condition and the one in which the mucous membrane shows coagulative necrosis and thrombi, and the submucosa an abscess-like condition from a great accumulation of leucocytes. Whenever the submucosa is markedly necrotic it is more or less densely infiltrated with leucocytes, which show, moreover, a decided tendency to collect in two layers—one being in relation to the muscularis mucosæ, the other in relation to the circular muscle. Both of these structures may be invaded with leucocytes. On the other hand, the submucosa may show so great an accumulation of leucocytes of the polymorphonuclear variety that the appearance suggests the formation of an abscess and yet the mucous membrane may be intact. The leucocytes show advanced necrotic changes, but the intercellular tissue does not undergo softening. A true abscess is, therefore, not formed.

The tendency of the pathological changes in the mucosa and submucosa to appear with greatest intensity in the spiral folds is clearly seen in the microscopical sections. The lesions diminish in intensity as the level surface of the mucosa is reached. While the entire depth of the mucosa in the full length of the fold may show coagulative necrosis, or hæmorrhage with disintegration or œdema, as the lower margins are approached the lesions extend less in depth and become discontinuous, until at the level of the mucosa microscopical areas, chiefly superficial ones, of necrosis or hæmorrhage appear in the mucosa, and the sub-mucous infiltration is disappearing. On the surface and within the substance of the necrotic mucous membrane a definite fibrinous and leucocytic pseudo-membrane may form. In this and in the adjacent necrotic membrane a massive bacterial development may take place.

While it is not our intention to describe the histological character of mercury colitis it is proper to state that a study of these lesions has shown us that they differ from those caused by dysentery toxin only in intensity. In localization and in character they resemble the severer type of lesions just described.

#### SUMMARY.

1. The facts described in this paper are intended to bear upon the pathogenesis of colitis in man and animals.

2. The toxin of the Shiga dysentery bacillus is liberated from the bacillus through the process of autolysis.

3. In rabbits the toxin is not absorbed directly in an active form by the gastro-intestinal tract; in man, however, absorption of an active poison does take place from the intestine.

4. The toxin is excreted in rabbits, and probably in man as well by the intestine, chiefly probably by the large intestine, which being injured by the act of elimination, reacts by the development of inflammation, etc.

5. In rabbits the characteristic action of the toxin depends upon the integrity of the biliary secretion into the intestine. When the bile is prevented from entering the intestine, either by ligature and section of the duct or by establishment of a biliary fistula, no lesions whatever of the large intestine appear, or they are inconsiderable in extent.

6. The loss of toxin through a biliary fistula does not prevent in rabbits the lethal effects which are caused, apparently, by a nervous poison. The tying off of the bile duct seems to prevent the passage of the toxin, in amounts sufficient to cause the large intestinal lesions, into the blood. The liver, therefore, in this condition tends to hold back the toxin from the general circulation.

7. The peculiar effects of the toxin on the large intestine in rabbits is not produced at once, but would appear to depend upon successive acts of excretion of the poison by the bowel.

8. The establishment of biliary fistula reduces the intensity of action of corrosive sublimate upon the large intestine in rabbits; and the lesions of ricin poisoning in these animals are also modified by this operation.

9. Dysentery toxin is destroyed by peptic digestion, and also, though probably more slowly, by tryptic digestion. The absence of power of the toxin to cause poisoning in rabbits when it is brought directly into the lumen of the intestine, is not explained by the destructive action of trypsin.

10. The character of the histological changes in the cæcum of rabbits caused by the dysentery toxin points to an action upon the substance and not primarily upon the surface of the intestine.





SOLUTION OF TISSUE WITH ABSCESS.

By EUGENE L. OPIE, M.D.

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In a former publication<sup>1</sup> I have shown that the two types of cells which especially act as phagocytes, namely the polynuclear leucocytes with fine granulation and the large mononuclear phagocyte or macrophage of Metschnikoff, are each characterized by a distinct proteolytic enzyme. That enzyme which is contained in the polynuclear leucocytes and as I have previously shown<sup>2</sup> is manufactured in the bone marrow is capable of causing proteolytic digestion in the presence of a neutral or alkaline reaction and is almost wholly incapable of action when placed in an acid medium. For this enzyme I have suggested the name leuco-protease, since it is characteristic of leucocytes contained in pus and other inflammatory exudates. The large mononuclear phagocytes of inflammatory exudates are especially concerned with the ingestion and destruction of cellular elements; the same cells are found in lymphatic glands adjacent to the site of inflammation and are doubtless identical with those which, for example, are abundant in the spleen lymphatic glands and other situations with typhoid fever. These cells contain an enzyme which is incapable of digesting proteid in an alkaline medium but is active in the presence of a weak acid; I have suggested for this enzyme the name lympho-protease.

The serum of an exudate produced by injecting aleuro-nat into the pleural cavity of a dog inhibits the proteolytic activity of leuco-protease<sup>3</sup>. The serum of the blood has the same property. Since this enzyme contained in the polynuclear leucocytes is incapable of digesting proteid in the presence of the blood serum its action is limited to these cells;

<sup>1</sup> *Jour. Exper. Med.*, 1906, viii, 410.

<sup>2</sup> *Ibid.*, 1905, vii, 316.

<sup>3</sup> *Ibid.*, 1905, vii, 759.

it can act only upon substances which have been ingested by these phagocytes and thus removed from the influence of the serum. Doubtless the same property of the serum serves to protect the tissues from the proteolytic enzyme contained in the polynuclear leucocytes, for even should it be set free by disintegration of these cells or otherwise its activity would be inhibited. Since lympho-protease does not cause proteolysis in a neutral or alkaline medium, the alkalinity of the blood serum would check its action should it be set free by disintegration of mononuclear phagocytes.

An experiment in which increasing quantities of serum are allowed to act upon the same quantity of enzyme demonstrates very clearly this anti-enzymotic property of the serum. Previous experiments have shown that the cells of an exudate obtained by injecting aleuronat into the pleural cavity of a dog preserve their power to cause proteolysis when dried after treatment with absolute alcohol and ether. Leuco-protease alone is preserved by this method. A weighed quantity of the dry enzyme has been allowed to act during five days at 37° C. upon a measured quantity of diluted blood serum (10 c.c.) heated to 75° C. for one half-hour and thus partially coagulated and denaturalized. The methods employed have been described in previous articles.

Nitrogen in substances uncoagulable by heat has been estimated by the Kjeldahl method and for convenience is represented by cubic centimeters of 1/10 N sulphuric acid.

20 mgr. powdered leucocytes	+	coagulated proteid			24.2 c.c.
20 " " "	+	" "	+	0.25 c.c. serum	22.15 "
20 " " "	+	" "	+	0.5 " "	18.8 "
20 " " "	+	" "	+	1.0 " "	10.6 "
20 " " "	+	" "	+	2.5 " "	7.55 "
Control, 2.85 c.c.					

Two and a half cubic centimeters of serum has almost completely inhibited the proteolytic action of twenty milligrams of the powdered cells (by subtracting nitrogen in uncoagulable form contained in the control mixture, digestion is found to be represented by 3.28 c.c.) while the addition of only a quarter of a cubic centimeter materially hinders digestion.

By adding increasing quantities of the enzyme-containing powder to the same quantity of serum it has been found that a given quantity of serum can inhibit the action of only a limited quantity of enzyme.

10 mgr. powdered leucocytes	+	coagulated serum	+	2.5 c.c. serum	6.15 c.c.
20 " " "	+	" "	+	2.5 " "	6.5 "
40 " " "	+	" "	+	2.5 " "	10.4 "
80 " " "	+	" "	+	2.5 " "	19.2 "
160 " " "	+	" "	+	2.5 " "	25.15 "

Nitrogen in uncoagulable form in coagulated serum + 2.5 c.c. serum is represented by 6.0 c.c.; in 160 mgr. powdered leucocytes, by 2.4 c.c.

The enzymotic activity of the powder used in the preceding experiment is shown by the following test:

20 mgr. powdered leucocytes	+	coagulated serum	18.9 c.c.
Control			4.3 "

Two and a half cubic centimeters of serum have completely inhibited twenty milligrams of the dried cells but have failed to prevent proteolysis when a larger quantity of enzyme is employed.

In a former publication I have shown that exudates removed from the pleural cavity of a dog one or two days after the injection of aleuronat fail to undergo autolysis because the anti-enzyme of the serum inhibits the proteolytic enzymes of the leucocytes. In exudates removed three days after injection of the inflammatory irritant slight autolysis occurs. Variation in the enzymotic activity of the serum of the exudates doubtless explains the inconstant results of Schutz<sup>4</sup> and of Zak,<sup>5</sup> who failed to find in human exudates any relation between the autolysis of an exudate and the abundance of its cellular elements.

Since an abscess is characterized by softening and solution of tissue it has seemed not improbable that the fluid obtained by centrifugalization from purulent exudates would fail to exhibit the anti-enzymotic action which is characteristic of sterile exudates produced in the pleural cavity by aleuronat, and, doubtless, according to the previously mentioned observations of Schutz and of Zak, of certain human exudates. As the result of the experimental pleurisy produced by aleuronat, at least, the chest wall is uninjured and the pleural cavity, save for a few adhesions, may return to a normal condition.

<sup>4</sup> *Cent. für inner. Med.*, 1902, xxiii, 1161.

<sup>5</sup> *Wiener klin. Woch.*, 1905, xviii, 376.

A sterile purulent exudate was obtained by injecting a small quantity (one cubic centimeter) of turpentine into the subcutaneous or intermuscular tissue of the flank of the dog. At the end of four or five days a large cavity distended with fairly thick purulent fluid is formed. Such purulent fluid contains polynuclear leucocytes in immense number together with mononuclear cells, fat globules, and particles of degenerated cells. Agar-agar inoculated with this purulent exudate has in every instance remained sterile. A measured quantity (5 c.c.) of this exudate was diluted with four times its volume of 0.85 per cent. sodium chloride and after addition of toluol subjected for five days to a temperature of 37° C.

5 c.c. pus at 37° C. for 5 days	12.6 c.c.
Control	7.05 "
Digestion	<hr/> 5.55 "

The following experiment demonstrates that the same purulent exudate is capable of digesting coagulated proteid under the conditions just mentioned.

2.5 c.c. pus + 5 c.c. coagulated serum	17.45 c.c.
Control	6.4 "
Digestion	<hr/> 11.05 "

In order to determine if the proteolysis caused by the purulent exudate is due to the absence of anti-enzyme in the serum of the exudate, cells were separated from the serum by centrifugalization. A cloudy fluid containing globules of fat but no leucocytes was obtained. The effect of this serum upon a given quantity of enzyme preserved as a dry powder was compared with the anti-enzymotic action of the blood serum from the same animal. In the following experiments the dry enzyme was prepared from the cells of a purulent exudate obtained after injecting turpentine into the subcutaneous tissue of a dog. Twenty milligrams of the powdered leucocytes were allowed to act upon coagulated serum in the presence of 2.5 c.c. of serum of the pus from which the dried enzyme was obtained or in the presence of serum of the blood of the same animal.

	Serum of pus	Serum of blood
With 2.5 c.c. of serum	14.15 c.c.	5.15 c.c.
The proteolysis produced by the same quantity of enzyme acting upon the same quantity of proteid without addition of serum is represented by 16.5 c.c.		

The following experiment, in which the conditions do not differ from those just described save that the preserved enzyme was obtained from an exudate produced by injecting alcuronat into the pleural cavity, further demonstrates the almost total absence of anti-enzymotic action in the serum from the abscess.

	Serum of pus	Serum of blood
With 1.5 c.c. of serum	17.0 c.c.	9.6 c.c.
With 2.5 c.c. of serum	17.6 "	9.4 "
Proteolysis produced by the same quantity of enzyme alone is represented by 17.95 c.c., the control containing nitrogen in uncoagulable form represented by 3.95 c.c.; 2.5 c.c. of serum of pus contains nitrogen in uncoagulable form represented by 2.1 c.c.; 2.5 c.c. of blood serum, by 1.7 c.c.		

A similar experiment in which serum from pus obtained five days after the injection of turpentine was used, confirms those just described. A measured quantity of coagulated proteid was subjected to the action of twenty milligrams of powdered leucocytes in the presence of one and of two and a half cubic centimeters of serum of pus or, for the sake of comparison, of blood.

	Serum of pus	Serum of blood
With 1. c.c. of serum	17.6 c.c.	6.7 c.c.
With 2.5 c.c. of serum	18.65 c.c.	7.35 "

Proteolysis produced by the same quantity of enzyme without the addition of serum is represented by 15.65 c.c., the control containing uncoagulable nitrogen represented by 4.3 c.c.; 2.5 c.c. of serum of pus contains nitrogen in uncoagulable form represented by 3.3 c.c., and the same amount of serum of blood by 1.7 c.c.

When allowance is made for the quantity of nitrogen in uncoagulable form in the various mixtures before digestion, it is evident that one cubic centimeter of blood serum completely inhibits the activity of twenty milligrams of the powdered leucocytes, but more than twice as much serum from the purulent exudate has no inhibitory action.

Since the purulent exudates used in the experiments just described have been obtained by injecting turpentine into the

subcutaneous tissue, it is possible that the absence of anti-enzyme in the serum of these exudates may be due to the action of this substance. In the following experiment the ability of 2.5 c.c. of serum to inhibit the digestion of coagulated proteid caused by forty milligrams of powdered leucocytes was tested in the presence and in the absence of turpentine. For the sake of comparison the same amount of enzyme was allowed to act upon coagulated proteid with and without turpentine in the presence of 2.5 c.c. of blood serum heated to 75° C in order to destroy its anti-enzymotic activity:

	Without turpentine	With turpentine
With 2.5 c.c. heated serum	31.2 c.c.	28.05 c.c.
With 2.5 c.c. serum	5.55 "	5.4 "
Control	3.55 "	4.3 "

Leuco-protease is capable of causing proteolysis in the presence of turpentine and the power of the blood serum to inhibit this digestion is unaffected by turpentine.

It is not improbable that an increasing quantity of proteolytic enzyme set free by disintegration of polynuclear leucocytes has so far overcome the anti-enzymotic action of a limited quantity of exuded serum that the entire pus is capable of active autolysis and has the power of digesting foreign proteid. The limited ability of a given quantity of blood serum to inhibit the action of increasing quantities of enzyme, demonstrated by the experiment already described, gives confirmation of this view. A purulent exudate is characterized by its ability to dissolve fibrin and necrotic tissue because its serum, unlike that of the non-purulent exudate, does not check the activity of the proteolytic enzyme furnished in great abundance by the polynuclear leucocytes.





## THE INFLUENCE OF COLLOIDS UPON THE DIFFUSION OF HÆMOLYSINS.

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PLATES XXVI, XXVII, XXVIII, XXIX, AND XXX.

The study which forms the subject of this paper was suggested by the experiments made by one of us (Flexner<sup>1</sup>) upon the restraining influence of certain colloids upon the injurious action of bile salts upon the pancreas. The results of the present work not only confirm the view there expressed, namely, that colloidal substances reduce the degree and rapidity of diffusion of certain crystalloids, but they would seem to have even a wider biological bearing. It is for these reasons that they are given, in some detail, in this place. In order that the diffusion of very complex molecules (such as certain hæmolysins, etc.) might be studied it was necessary to devise a method of testing diffusion that would give approximately accurate results without having to resort to chemical analyses which are, indeed, useless with many of these bodies. We found that a suspension of blood corpuscles in certain colloids supplied a useful and accurate means of determining the rate of diffusion of simple and complex molecules which bring about hæmolysis. By reversing the experiment, namely by placing the chemical bodies in the colloids the rate of diffusion into water and saline could also be determined by estimating the quantity of the hæmolytic substance which appeared in these fluids after a given time.

The earlier studies on the influence of colloids upon the rate of diffusion showed, for the most part, that the rate remained unchanged. Graham<sup>2</sup> believed that no inhibition followed from the presence of colloids. He dissolved, among other things, 10 per cent. of sodium chloride in a 2 per cent. agar-agar jelly and overlaid this in a tube with another portion of the jelly

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<sup>1</sup> *Jour. Exp. Med.*, 1906, viii, 167.

<sup>2</sup> *Phil. Trans. Roy. Soc.*, 1861, cli, Part I, 183; *Liebig's Annalen*, 1862, cxxi, 1.

minus the salt. At the end of eight days the amount of salt which diffused into the plain agar was found to be the same as that diffused into water. De Vries<sup>3</sup> failed to notice any marked difference in the diffusion of solutions of potassium chromate and copper sulphate into pure water and 4 per cent. silicic acid after a contact of four months. Voigtländer<sup>4</sup> investigated the rate of diffusion of certain simple acids and salts into agar-agar of from 1 to 4 per cent. While the rate of diffusion was at first in favor of the weaker concentration the actual quantities of salts and acids which diffused were about the same in all cases. Reformatsky<sup>5</sup> observed no inhibition of the catalytic reaction of acetate of methyl and hydrochloric acid by a 1.25 per cent. agar suspension; and Levi<sup>6</sup> ascertained that 1.57 per cent. silicic acid, 1 per cent. agar, and 0.6 per cent. gelatin did not alter electric conductivity, lower the freezing point, or inhibit the inversion of cane sugar by hydrochloric acid.

Not all experimenters have found that colloids act in a wholly indifferent manner upon the process of diffusion. Stefan<sup>7</sup> recalculated the values of Graham's experiments and ascertained that the diffusion constant was smaller with agar-agar jelly than with pure water. Chabry<sup>8</sup> found that gelatin and cartilage delayed the action of acids on orcein. While our own experiments were in progress a paper appeared by Kurt Mayer<sup>9</sup> in which the inhibiting action of gelatin, agar, and egg white upon diffusion is described. The bodies employed by him—sodium chloride and potassium chromate—were simple, and his results point to an unmistakable retardation of diffusion by colloids, the retardation being approximately proportional to the concentration of the colloids. With agar the differences are less marked than with gelatin, on account of the wider variations in concentration practicable with the latter substance. Similar results were obtained by Nell<sup>10</sup> who studied the effect of gelatin on the diffusion of a number of chemical bodies. Nell also observed that the electrical conductivity of solutions of copper sulphate are reduced by gelatin when present in amounts of from 1 to 20 per cent.

The number of substances the diffusion of which can be satisfactorily observed by the hæmolytic method is considerable. We studied acids, alkalies, sodium taurocholate, saponin, solanin, cobra venom, and tetanus toxin. In the case of the complex bodies, snake venom and tetanus toxin, the capacity for diffusion of the different constituents, namely, hæmolysins, neurotoxin, tetanospasmin, was also determined.

<sup>3</sup> *Recueil des travaux chimiques de Pays-Bas*, 1884, iii, 375.

<sup>4</sup> *Zeitschr. f. physik. Chemie*, 1889, iii, 316.

<sup>5</sup> *Zeitschr. f. physik. Chemie*, 1891, vii, 34.

<sup>6</sup> *Gazetta chimica italiana*, 1900, xxx, Parte II, 64.

<sup>7</sup> *Wiener Sitzungsberichte*, 1879, lxxix, II Abt., 161 and 215.

<sup>8</sup> *Journal de Physique*, 1888, vii, 115.

<sup>9</sup> *Beiträge z. chem. Physiologie u. Pathologie*, 1905, vii, 393.

<sup>10</sup> *Annalen d. Physik*, 1905, IV Folge, xviii, 323.

The particular points which were noted were: the influence of concentration of colloids upon the rate of diffusion; the time relations of the rate of diffusion; and the comparative degree of diffusibility of different substances.

The diffusible bodies were made to diffuse (1) from saline solution into colloids, (2) from colloids into colloids, and (3) from colloids into saline solution.

#### METHODS.

In preparing the various solutions and blood mixtures a fair degree of asepsis was observed. Where the experiments extended over any length of time greater precautions were taken. The blood was obtained aseptically and the colloids were sterile. The blood mixtures with agar and gelatin were well preserved at the end of a week or even longer. The experiments were made at room temperature which was about 20° C. The agar suspensions were made with agar-agar which had been thoroughly washed in running water. The agar was dissolved in distilled water, clarified, filtered, and 0.9 per cent. sodium chloride added. The gelatin was dissolved in distilled water, neutralized with NaOH, clarified, filtered, and salt in 0.9 per cent. added.

The highest concentrations of the colloids having been made, the weaker strengths were obtained by the addition of 0.9 per cent. salt solution. Acids, alkalies, and salts were made into normal solutions. These solutions were further diluted according to the hæmolytic strength of the different chemical bodies. In making the mixtures of chemical body and colloid a concentrated solution of the first was added to the second so as to avoid undue dilution of the colloid.

The colloids were pipetted into tubes of 8 mm. to 15mm. diameter, care being taken to prevent flowing over the wall of the tube above the layer. The quantities employed were, usually, 2 c.c. each of colloid and fluid. The colloid column measured from 32 to 33 mm. in tubes of 8 mm. diameter.

Dog and rabbit blood in 3 to 5 per cent. suspensions in 0.9 per cent. salt solution were used. The blood and colloid were mixed at low temperatures after which they were congealed. Ice was used to congeal the gelatin blood mixtures.

The readings were made with a scale one degree of which was 0.7 mm. All the figures were subsequently reduced to millimeters.

In determining the quantity of hæmolytic or toxic body which diffused from the colloid into the supernatant saline the latter fluid was removed at intervals and the hæmolytic or toxic strength determined. These values could be expressed in hæmolytic and in toxic units.

#### DIFFUSION OF HÆMOLYSINS FROM FLUID INTO COLLOID MEDIA.

There is no difficulty in observing the extent to which the hæmolytic body has penetrated into the blood-colloid suspension since

the originally opaque mixture becomes perfectly transparent as the result of the laking of the corpuscles. The line of penetration is usually sharp and straight, but in some cases it is irregular.

*Series I.*—Hydrochloric, nitric, oxalic acids, sodium hydroxide, and sodium carbonate in  $\frac{1}{10}$  N. solutions were employed. The figures for hydrochloric and nitric acids are almost identical, although the degree of diffusion of hydrochloric is very slightly higher than of nitric acid. The values in millimeters for hydrochloric acid at the different periods (h. equalling hours) are as follows:

	9 h.	18 h.	42 h.	60 h.	84 h.
25% gelatin	4.5	7.7	13.0	16.5	18.2
10% "	5.4	9.1	16.2	20.	22.4

The values for oxalic acid are lower:

	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	2.75	4.9	9.1	11.2	12.6
10 % "	3.4	6.3	12.3	14.0	16.8

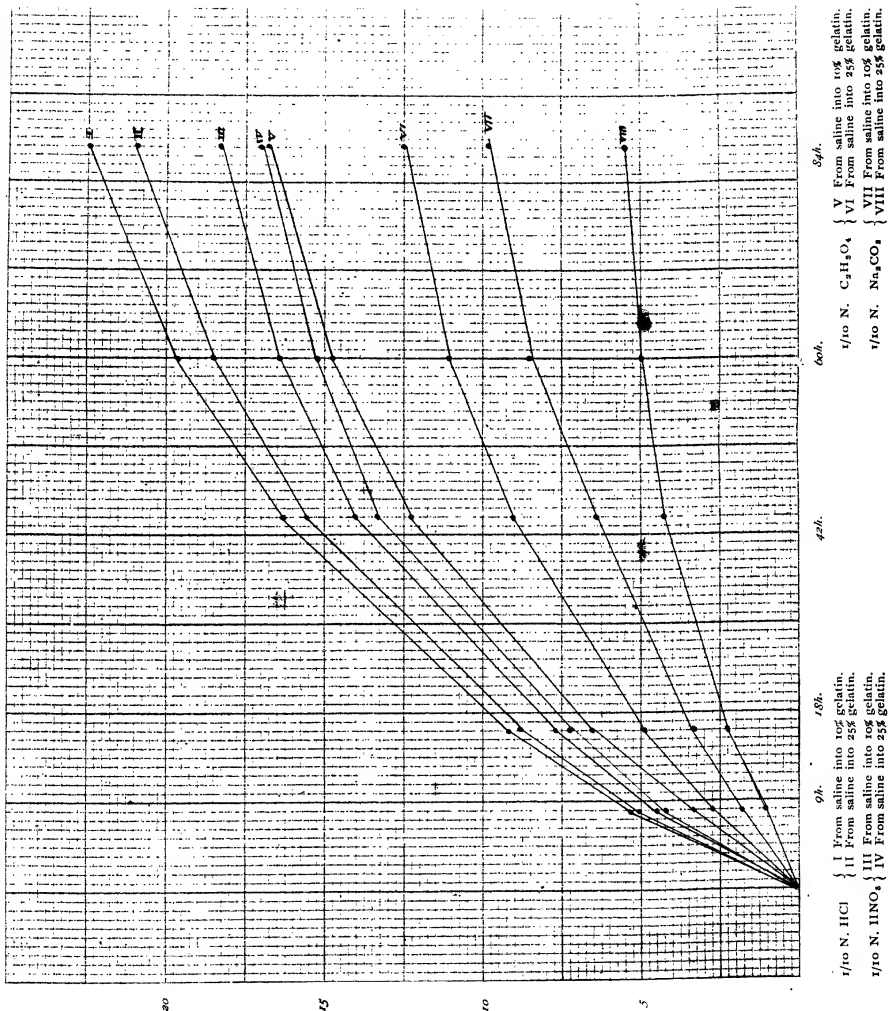
And the values for sodium carbonate are still lower:

	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	1.25	2.24	4.2	4.9	5.6
10 % "	1.75	3.3	6.5	8.4	9.8

The comparative rate of diffusion into gelatin of these simple bodies can be readily expressed graphically in the form of a curve (Plate XXVI). The influence of concentration of colloid on the rate and degree of diffusion is clearly seen in the curve. Lines I and II indicate the diffusion of hydrochloric acid into 10 per cent. and 25 per cent. gelatin respectively. Lines III and IV indicate nitric acid, V and VI oxalic acid, and VII and VIII sodium carbonate. The black dots show the reading periods and the marginal figures stand for millimeters.

On comparing the diffusion values for 10 and 25 per cent. gelatin the relation can be expressed by the following formula: *The amount of diffusion is approximately inversely proportional to the square root of the concentration of the gelatin.* The deviations from this formula can possibly be accounted for by considerations of experimental error, impurity in the gelatin, and the resistance introduced by the erythrocytes. The velocity of diffusion decreases steadily with increase in time.









The concentration limits for agar are smaller than for gelatin and consequently the differences in degree and velocity of diffusion are also smaller with that colloid. The strengths employed in the next experiments were 0.5, 1, and 2 per cent. Two examples only, namely of nitric acid and sodium hydroxide, will be given.

HNO<sub>3</sub>  $\frac{1}{10}$  N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.8	9.75	13.5	18.75	21.5	24.6
0.5% "	5.95	9.8	13.8	19.0	21.8	24.8

NaOH  $\frac{1}{10}$  N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.6	9.5	13.5	18.5	21.5	24.5
0.5% "	5.95	9.6	13.75	18.75	21.5	28.

*Series II.*—In the next experiments sodium taurocholate, saponin, and solanin were employed. The hæmolytic values were first determined in the ordinary manner. They can be expressed for rabbit blood in the following figures: 0.0001 (saponin), 0.0007 (solanin), 0.007 (sodium taurocholate). The value of hydrochloric acid, under the same conditions was 0.02.

Sodium Taurocholate  $\frac{1}{10}$  N. Rabbit Blood 5%

	3 h.	6 h.	12 h.	24 h.	42 h.	60 h.	84 h.	
25% gelatin	1.0	2.0	3.15	4.3	5.6	7.0		1st determination.
		1.2	2.2	3.9	5.6	7.0	8.4	2d "
10% "	1.4	2.6	4.3	6.6	7.8	9.0		1st "
		2.0	3.5	5.6	7.7	9.3	11.2	2d "

Saponin  $\frac{1}{100}$  N. Rabbit Blood 5%

	3 h.	6 h.	24 h.	48 h.	
25% gelatin	1.0	2.0	4.2	6.3	1st determination.
		2.4	5.0	7.0	2d "
10% "	1.4	2.9	6.6	9.15	1st "
		2.85	6.0	8.4	2d "

Solanin  $\frac{1}{100}$  N. Dog Blood 3%.

	6 h.	24 h.	48 h.
25% gelatin	3.6	7.5	10.5
10% "	4.6	9.0	13.5

The relation which exists in the case of gelatin, between the degree of diffusion and the concentration of the colloid is, as this

experiment shows, the same for certain chemicals of high and of low molecular weight. When agar-agar is substituted for gelatin the differences are less marked, although a difference can still be made out. Taking saponin as an example the following figures are obtained:

		Saponin $\frac{1}{100}$ N.					
		3 h.	6 h.	24 h.	48 h.	1st determination.	
1%	agar	2.45	5.0	9.8	14.0	"	"
0.5%	"	3.85	8.4	17.5	24.5	"	"
1%	"	1.7	3.5	8.9	10.5	2d	"
0.5%	"	1.8	3.8	9.3	11.2	"	"

*Series III.*—The next experiments were made with the complex lysins of cobra venom and tetanus toxin. Two specimens of cobra venom were available: one a fresh solution of 0.4 per cent., one cubic centimeter of which contained for dog blood 2000 minimal hæmolytic doses; and a second solution of the same strength which had been on ice seven months and was ten times weaker. The samples of tetanolysin contained 33 minimal hæmolytic doses per cubic centimeter.

The experiments with dog blood and 0.1 per cent. cobra venom were not wholly satisfactory, since the line of demarkation was not always sharp. The influence of concentration of colloid is, however, shown even in this case.

		3 h.	9 h.	24 h.
25%	gelatin	0.84	2.1	6.0
10%	"	2.1	5.6	31.5

The next experiment was made with cobra venom, lecithin, and rabbit corpuscles, in which case the diffusion is regular.

Cobra Venom 0.2% 1 c.c., Lecithin  $\frac{1}{100}$  N. 1 c.c.

		12 h.	24 h.	42 h.	60 h.	84 h.
25%	gelatin	1.7	2.8	4.20	4.9	5.6
10%	"	1.8	3.15	4.55	5.55	6.3

The employment of the old cobra venom gave, instead of a curve, an almost straight line.

Passing now to the results with cobra venom 0.1 per cent. and dog blood in agar, definite inhibition in the higher concentration is found to occur.

	6 h.	24 h.	48 h.
2% agar	1.4	5.25	9.1
0.5	2.1	7.0	12.6

Cobra venom diffuses into agar more readily than into gelatin. The rate of diffusion into agar is proportional not to the square root of the time but almost directly to the time.

Tetanolysin penetrates gelatin (strength below 10 per cent.) more quickly than it does agar of 0.5 per cent., which makes an exception to the rule.\*

#### DIFFUSION OF HÆMOLYSINS FROM ONE COLLOID INTO ANOTHER.

The next experiments were designed to show whether any further inhibiting effect would be developed if the hæmolysin were enclosed in the colloid. Since the velocity of diffusion is smaller the higher the concentration of the colloid, it was presumable that the introduction of a second colloid medium would further reduce the velocity.

Sodium taurocholate, saponin, solanin, and pyrogallie acid were studied. In the first experiment to be given sodium taurocholate in  $\frac{1}{10}$  N. solution was enclosed in gelatin of 10 and 25 per cent., and dog blood of 3 per cent. was suspended in agar of 0.5 and 2.0 per cent.

#### 2% Agar and Dog Blood.

Sod. taurocholate in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.55	2.4	3.15	4.0	5.6
10% "	1.7	2.6	3.6	4.9	7.0
Saline (control)	2.55	3.55	5.15	7.3	9.8

\* Arrhenius and Madsen (*Contributions from the University Laboratory for Medical Bacteriology, Copenhagen, 1902*) have applied the method of diffusion into gelatin to a determination of the molecular weight of diphtheria toxin. They ascertained that diphtheria and tetanus toxin diffused into 10 per cent. gelatin; that the former underwent no change in the process and the latter diffused more rapidly than the former. The corresponding antitoxins passed into the gelatin more slowly than the toxins.

Craw (*Proc. of the Royal Soc., Series B., 1906, lxxvii, 311*) determined that bacillus megatherium lysin passes more slowly through a porous filter impregnated with 15 per cent. than with 7.5 per cent. gelatin.

## 0.5% Agar and Dog Blood.

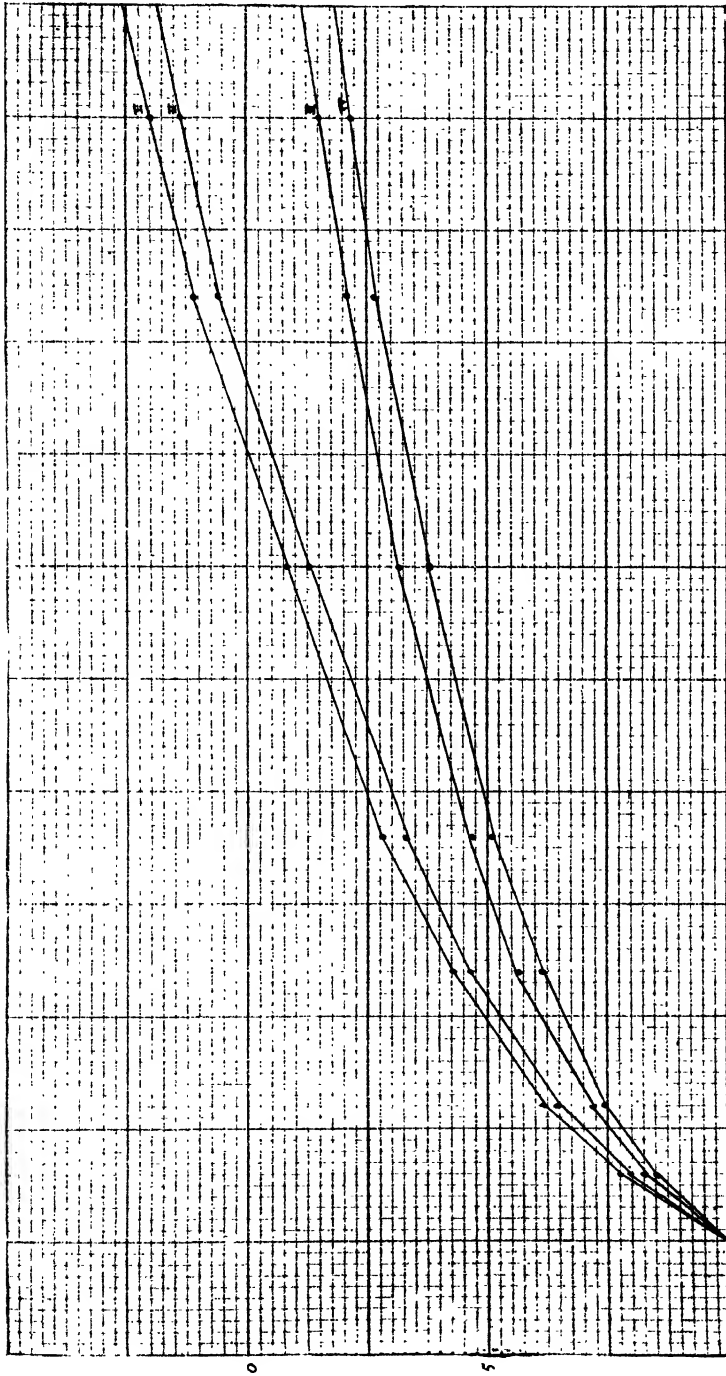
Sod. taurocholate $\frac{1}{10}$ in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.5	2.45	3.15	4.55	5.6
10% "	1.8	2.8	3.8	5.0	7.0
Saline (control)	2.6	3.85	5.2	7.3	9.8

Hence taurocholate of sodium diffuses from 25 per cent. gelatin into agar more slowly than it does from 10 per cent. gelatin. The difference in concentration of the agar has only a slight influence on the rate of diffusion. In a second experiment the salt was dissolved in agar and the blood suspended in gelatin. The influence of concentration (0.5 and 2.0 per cent.) of the agar was very small, and the diffusion from agar was slightly greater than from saline solution (control).

The experiments with  $\frac{1}{100}$  N. saponin dissolved in agar and dog blood suspended in gelatin showed diffusion to take place more rapidly into 10 than into 25 per cent. gelatin, and much more rapidly from agar than from the saline control. When, however, the colloids were reversed, namely, the blood suspended in agar and the solanin dissolved in gelatin, the influence of the concentration of the gelatin came out, the diffusion from the saline being greater than that from the colloid. Plates XXVII and XXVIII express these effects in the form of curves.

The explanation of this discrepancy is to be found, possibly, in the sum of inhibitions exerted by the gelatin and the corpuscles, of which the first is the greater; while, as has frequently appeared in these experiments, the greatly weaker agar concentrations exercise slight inhibition only. A similar effect, although a smaller one, is produced in the case of pyrogallie acid.

In the study of the more complex haemolysins of venom it was found, in spite of certain irregularities of diffusion, that (1) the velocity of diffusion from agar into the gelatin blood is slower in the case of 25 than in the case of 10 per cent. gelatin, (2) the haemolysin of cobra venom is diffused from agar into gelatine more slowly than from these colloids into saline, (3) concentrations of agar between 0.5 and 2.0 per cent. act about the same, and (4) the relation of time to diffusion does not correspond to Fick's law.



50h.

42h.

30h.

Saponin.

18h.

12h.

6h.

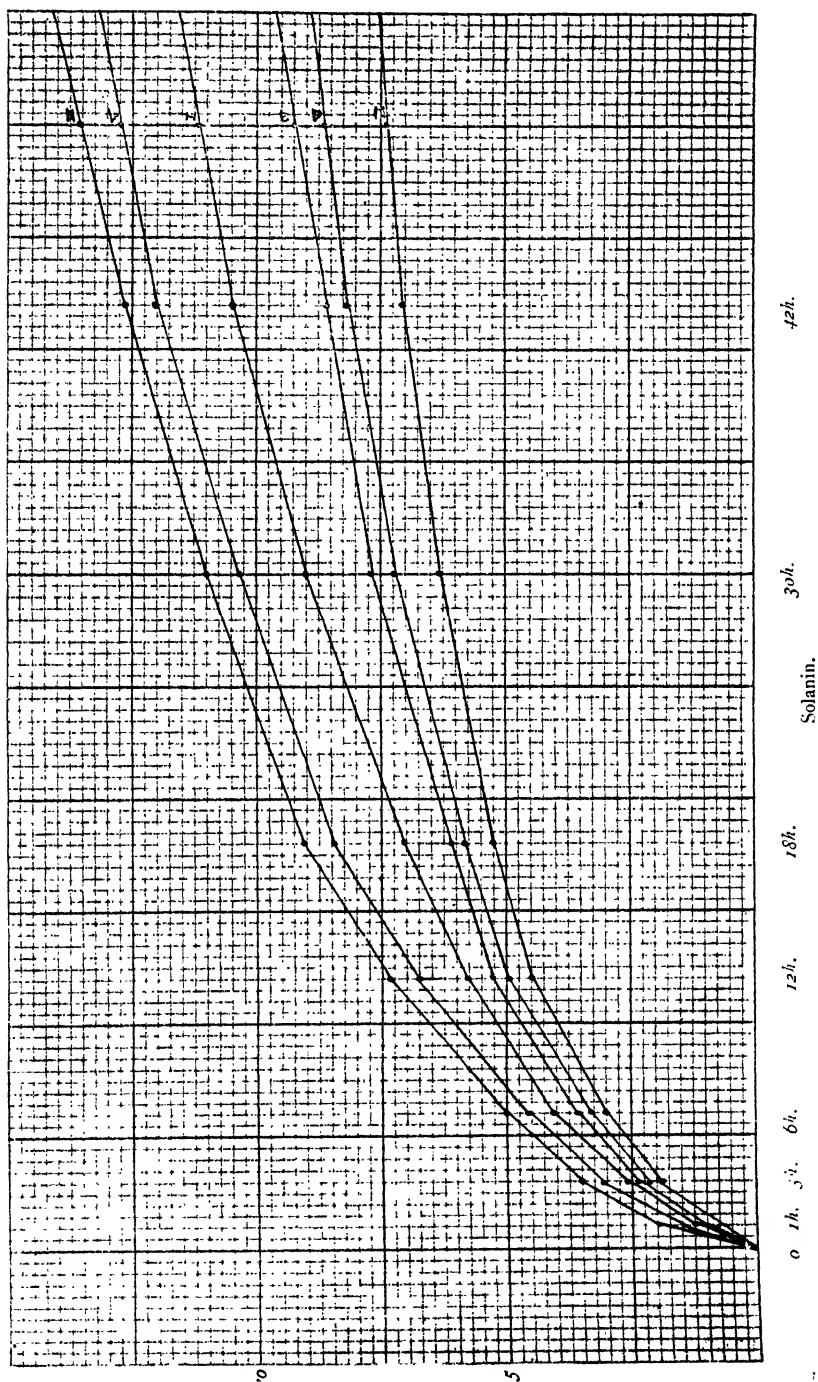
3h.

0

I From saline into 0.5% agar.  
II From saline into 1% agar.

III From 10% gelatin into 1% agar.  
IV From 25% gelatin into 1% agar.









## DIFFUSION FROM COLLOID INTO SALINE SOLUTION.

Sodium taurocholate, saponin, and solanin were dissolved in 10 and 25 per cent. gelatin and poured into test-tubes of 20 mm. diameter. Five cubic centimeters of the colloids were put into each tube after the congelation of which an equal quantity of saline solution was poured in. The saline fluid, after contact, was removed at stated periods, and the quantity of hæmolyisin present in it was estimated colorimetrically. Three

TABLE I.

SAPONIN  $\frac{1}{10}$  N.

Period of Contact	Degree of Hæmolyisis	25% gelatin		10% gelatin	
		Quantity in c.c. required to produce stated degree of hæmolyisis	Corresponding number of hæmolytic units in 1 c.c. of saline	Quantity in c.c. required to produce stated degree of hæmolyisis	Corresponding number of hæmolytic units in 1 c.c. of saline
1 hour	100%				
	30%	1.8	0.55	1.	1.
	5%	0.9	1.1	0.55	1.8
2 hours	100%				
	30%	1.4	0.7	0.6	1.6
	5%	0.7	1.4	0.5	2.
4 hours	100%				
	30%	0.75	1.33	0.5	2.
	5%	0.5	2.	0.35	2.8
24 hours	100%	0.7	1.4	0.5	2.
	30%	0.3	3.3	0.2	5.
	5%	0.18	5.5	0.14	7.1

SOLANIN  $\frac{1}{10}$  N.

1 hour	100%	0.55	1.8	0.33	3.
	30%	0.14	7.	0.09	11.
	5%	0.125	8.	0.085	12.
2 hours	100%	0.33	3.	0.25	4.
	30%	0.09	11.	0.075	13.3
	5%	0.085	12.	0.07	14.
4 hours	100%	0.25	4.	0.18	5.5
	30%	0.07	14.	0.055	18.
	5%	0.055	18.	0.04	25.
24 hours	100%	0.1	10.	0.07	14.2
	30%	0.03	33.	0.025	40.
	5%	0.025	40.	0.02	50.

grades were used: 100, 30, and 5 per cent. It was found that sodium taurocholate was largely held back for at least 48 hours by the gelatin, probably because of a chemical union between the salt and the proteid. Only a trace of hæmolysis was obtained at the end of this period. The results obtained with saponin and solanin are given in the accompanying table (Table I), and they show that the quantities of these bodies diffused from 25 and 10 per cent. gelatin bear the proportion of 1 : 1.25-1.40. Hence the velocity and amount of diffusion from colloid into saline, like that from saline into colloid, is inversely proportional to the square root of the concentration of the colloid.

INFLUENCE UPON THE RATE OF DIFFUSION OF DIAMETER OF SURFACE, DEPTH OF MEDIUM, AND CONCENTRATION OF HÆMOLYSIN.

TABLE II.

Diameter of Tube	Amount of		Period of Contact	1:100 N. Saponin		1:1000 N. Saponin		1:10000 N. Saponin	
	Gelatin Blood	Saponin Solution		25% gelatin	10% gelatin	25% gelatin	10% gelatin	25% gelatin	10% gelatin
8 mm.	4 c.c.	4 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	4 c.c.	2 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
15 mm.	2 c.c.	4 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	2 c.c.	2 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
15 mm.	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
15 mm.	1 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.5	9.1	7.	7.7	3.85	3.85

The substances used in these experiments were solanin and saponin in saline solution and dog blood mixed with gelatin. The results are given in Tables II and III and they show that no marked influence is exercised on the diffusion by diameter of surface and depth of medium. But other facts may be noted.

TABLE III.

Diameter of Tube	Amount of		Period of Contact	1:5 N. Solanin		1:100 N. Solanin		1:1000 N. Solanin	
	Gelatin Blood	Solanin Solution		25% gelatin	10% gelatin	25% gelatin	10% gelatin	25% gelatin	10% gelatin
15 mm.	4 C.C.	4 C.C.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
	4 C.C.	2 C.C.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.3	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 C.C.	4 C.C.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
8 mm.	2 C.C.	2 C.C.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 C.C.	1 C.C.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	5.9		
	1 C.C.	2 C.C.	6 hours	3.6	4.6		1.25		
			24 "	8.	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		

The relative capacity for diffusion of saponin and solanin from saline solution into gelatin blood is expressed in hæmolytic units by the proportion of 1:1.5. These figures are to be compared with those in which the hæmolysin is enclosed in the colloid (Table I) and allowed to diffuse into saline, in which series the solanin is shown to diffuse from seven to eight times more hæmolytic units in a given period than the saponin. But

as solanin is nearly twice as strong an hæmolytic agent as saponin the proportion of diffused hæmolytic units would stand, as regards diffusion from colloid into saline, as 1:4 against 1:1.5 for saline into colloid. The diffusion velocity for solanin is, under the former conditions, four times as great as for saponin; and the inhibiting influence of the colloid is greater when the crystalloid seeks to enter it from than when it attempts to leave it for a fluid medium.

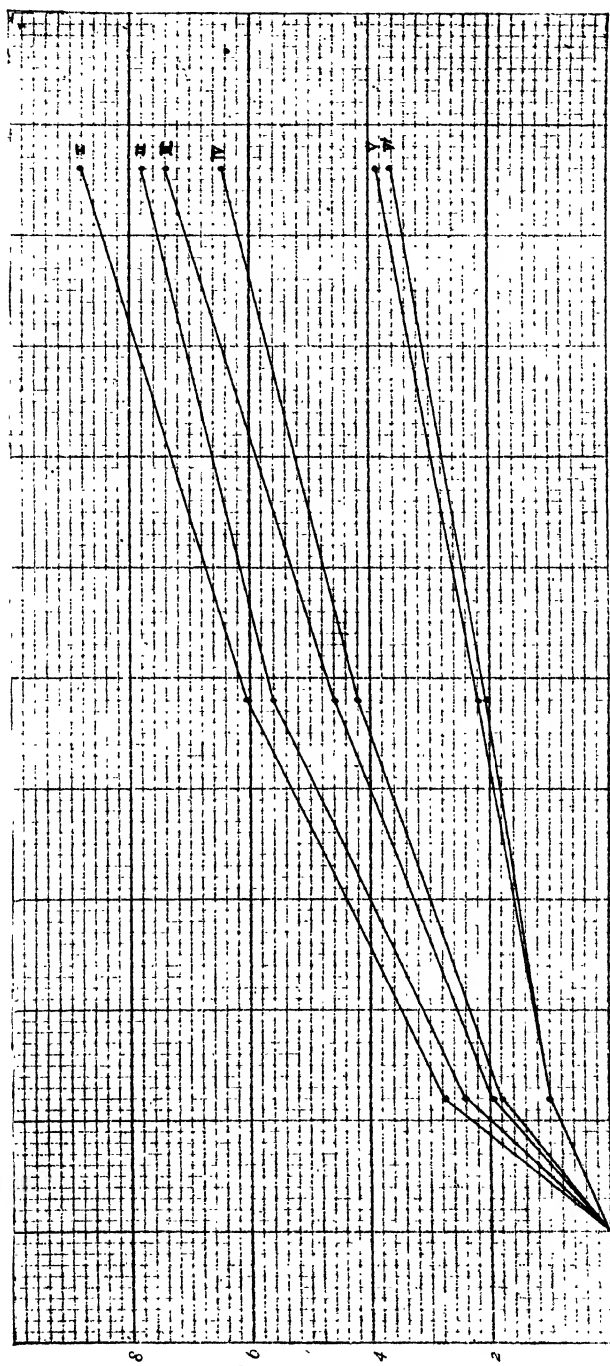
The concentration of the active substance plays a considerable part in relation to the rate of diffusion. The effects of this concentration are best seen by observing Plates XXIX and XXX. In the case of saponin while the difference in diffusion between  $\frac{1}{100}$  N. and  $\frac{1}{1000}$  N. is very slight, that between  $\frac{1}{100}$  N. and  $\frac{1}{10000}$  N. becomes as about 2:1. In the case of solanin the difference between  $\frac{1}{100}$  N. and  $\frac{1}{1000}$  N. is as 2:1, and between  $\frac{1}{100}$  N. and  $\frac{1}{10000}$  N. more than 10:1.

In Plate XXIX, the following values are given: I and II represent the degree of diffusion of  $\frac{1}{100}$  N. saponin from saline into 10 per cent. and 25 per cent. gelatin respectively; III and IV represent the degree of diffusion of  $\frac{1}{1000}$  N., and V and VI, the degree of diffusion of  $\frac{1}{10000}$  N. saponin under the same conditions. The values for Plate XXX are as follows: I and II represent the degree of diffusion of  $\frac{1}{100}$  N. solanin from saline into 10 per cent. and 25 per cent. gelatin respectively; and III and IV the degree of diffusion of  $\frac{1}{1000}$  N. solanin under the same conditions.

TABLE IV.

Scale of Hæmolysis*	Saponin				Solanin			
	25% gelatin		10% gelatin		25% gelatin		10% gelatin	
	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N
a	1.		0.6		0.25	2.	0.16	1.5
b	0.3		0.25		0.06	0.7	0.05	0.5
c	0.2	2.	0.15	1.5	0.045	0.5	0.035	0.35

\* The scale of hæmolysis was an arbitrary and descending one—a being highest and c lowest.



48h.

24h.

6h.

0

Saponin.

1/100 N. Solution { I From saline into 10% gelatin.  
II From saline into 25% gelatin.

1/10000 N. Solution { V From saline into 10% gelatin.  
VI From saline into 25% gelatin.

1/10000 N. Solution { III From saline into 10% gelatin.  
IV From saline into 25% gelatin.



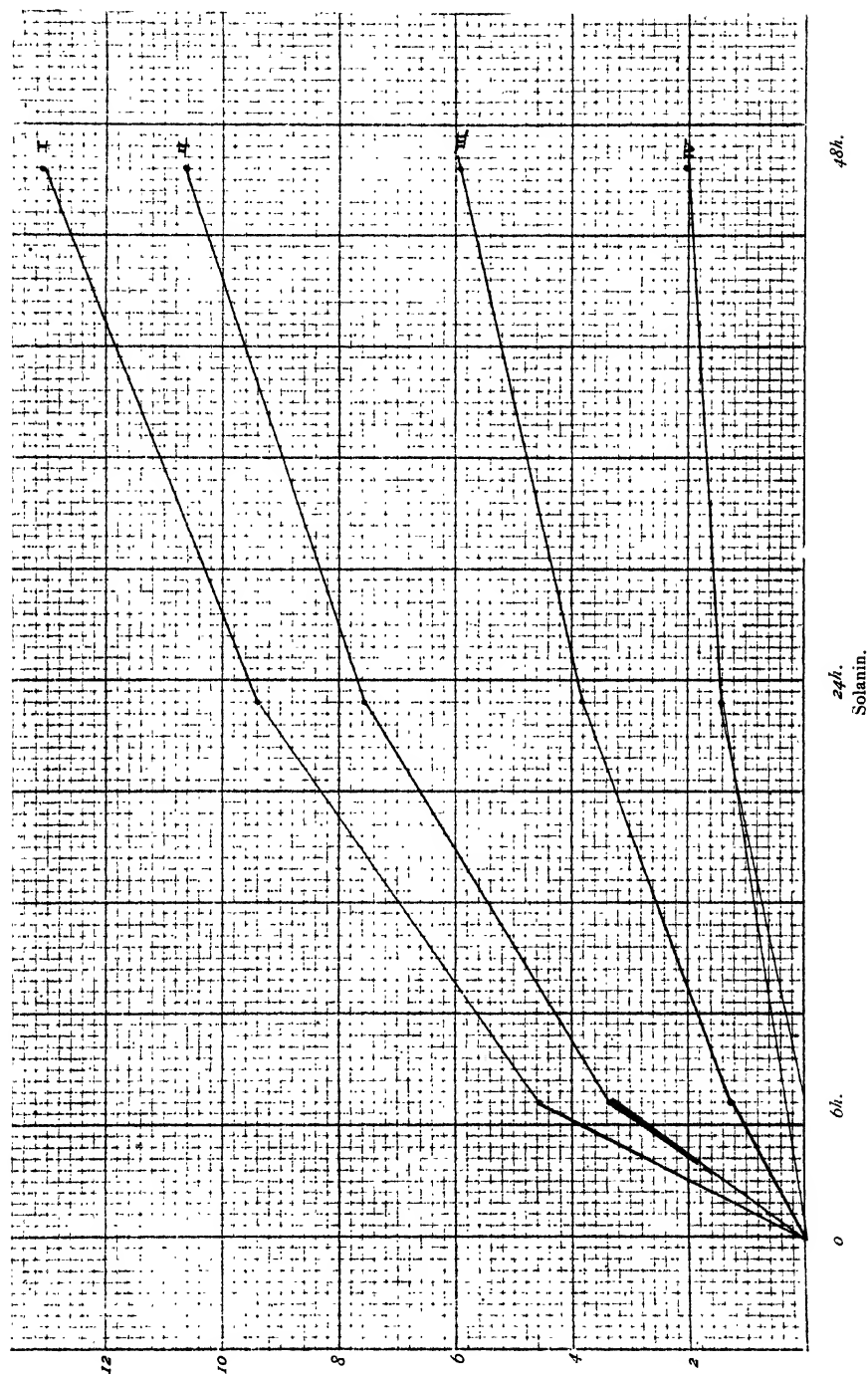






TABLE V.

Period of Contact	Degree of Hæmolysis	1:100 N. Solanin		1:1000 N. Solanin	
		25% gelatin	10% gelatin	25% gelatin	10% gelatin
1 hour	{ 30% 5%	0.14* 0.125	0.09 0.085	1.5 1.	1. 0.65
2 hours	{ 30% 5%	0.09 0.085	0.075 0.07	1. 0.7	0.8 0.55
4 hours	{ 30% 5%	0.07 0.05	0.055 0.04	0.7 0.5	0.6 0.4
24 hours	{ 30% 5%	0.03 0.025	0.025 0.02	0.03 0.25	0.25 0.2

\* These figures indicate the quantity of supernatant saline required to produce the degree of hæmolysis stated.

The differences noted are not entirely easy of explanation; and it is especially difficult to account for the behavior of solanin, in that while diffusing much more readily than saponin, it should, at the same time, be so greatly influenced by concentration. These considerations apply only to the special case in which the hæmolysins are contained in the saline; when they are enclosed in the colloid and allowed to diffuse into saline the amount of diffusion is directly proportional to the concentration of the hæmolysins.

#### DIFFUSION OF COBRA VENOM AND TETANUS TOXIN INTO COLLOIDS.

It has been stated that the hæmolysins of cobra venom and tetanus toxin pass from saline solution into gelatin and agar. A series of experiments was made to determine the effect of enclosing the toxins in one colloid (agar) and the blood in another (gelatin), and the reverse. Using a 5 per cent. suspension of dog blood in 10 per cent. gelatin and 0.1 per cent. cobra venom

in 2 per cent. agar, it was found that complete hæmolysis occurred in twenty-four hours, while when the blood was mixed with the agar and the venom with the gelatin, the hæmolysis proceeded much more slowly. It was also ascertained that the venom hæmolysin passes more quickly into gelatin from agar than from saline solution. In this respect it agrees with the more definite chemical hæmolysins.

The next question considered was whether the entire series of venom principles pass into the colloids, and if so whether they diffuse with equal velocity. To test this the hæmolysed portion of the gelatin- and agar-blood mixtures was carefully separated from the other in which the blood corpuscles were still intact, and each portion was injected into guinea-pigs to determine the toxicity. In the case of the gelatin-blood mixture the portion to be injected intraperitoneally was first melted, and in the case of agar it was emulsified in saline solution. The gelatin used was 10 per cent., and the agar 2 per cent. for enclosing the venom, and 0.5 per cent. for enclosing the blood.

Two cubic centimeters of the venom-colloid mixture were used in each experiment. The number of minimal lethal doses contained in this quantity, for guinea-pigs, of about 180 grams weight, was sixteen. It was ascertained that three m.l.d. of the venom passed in the hæmolysed portion from the agar into the gelatin blood, and one m.l.d. from the gelatin into the agar blood. It was further ascertained that the non-hæmolysed portion of the colloid-blood mixture does not contain the cobra neurotoxin in appreciable quantities. In other words, cobra lysin and cobra neurotoxin diffuse together.

Since the two principles diffuse together it was desirable to ascertain, if possible, whether they diffuse accurately in the proportion in which they exist in venom; that is, whether the whole venom passes unchanged into the colloid, or whether a separation of the components occurs. It has been stated that, using two cubic centimeters of a 0.1 per cent. cobra venom suspension in agar, one fifth of its toxic constituents passed into the gelatin-blood mixture in twenty-four hours. It was ascertained that two cubic centimeters of the venom mixture contained 600

complete hæmolytic doses of cobra lysin (for dog blood). Since the rate of diffusion of cobra lysin is very little affected by time, as it proceeds almost in a straight line, all that is required to determine the quantity of diffused lysin is to multiply the amount of diffusion by the time. It was found by experiment that an amount of cobra lysin necessary to hæmolyse two cubic centimeters of a 5 per cent. suspension of dog corpuscles will require from three to four hours to pass from 10 per cent. gelatin containing 0.1 per cent. venom to saline solution; and that in twenty-four hours about twenty-four complete hæmolytic doses will have diffused into the saline. Hence the rate of diffusion of cobra lysin is as 24:600 or 1:25. In the same time, as we have seen, the quantity of cobra neurotoxin which diffused from agar into gelatin was as 1:5. In other words, cobra neurotoxin diffused five times faster than cobra lysin from agar into a gelatin-blood mixture. This fact interesting in itself is rendered more suggestive in view of Faust's<sup>11</sup> recent statement of a relationship between cobra neurotoxin and the saponin substances.

A series of experiments similar to the above was made with a tetanus toxin one cubic centimeter of which contained 1000 minimal lethal doses for guinea-pigs of 250 grams and rats of 60 grams weight, and 33 minimal hæmolytic doses. It was found that both tetanolysin and tetanospasmin pass together easily into gelatin and agar, since the non-hæmolysed portions of the blood-colloid cylinder do not contain an appreciable quantity (less than 0.0001 c.c.) of the toxin. It would, however, appear from our experiments that tetanolysin diffuses more rapidly into colloids than tetanospasmin, since the great disproportion in quantity of spasmin and lysin—1000:33—which exists in the tetanus toxin does not bring about a corresponding preponderance of the spasmin in the colloid.

#### SUMMARY.

Acids, alkalies, salts, glucosides, and certain toxins diffuse

<sup>11</sup> Die tierische Gifte, Braunschweig, 1906, p. 54.

more quickly into 0.9 per cent. salt solution than into agar-agar and gelatin suspensions.

The inhibitory effect of the colloids grows with increase in concentration, which increase affects both the velocity and extent of the diffusion.

In the case of gelatin the degree of diffusion is approximately in inverse proportion to the square root of the concentration. Agar-agar in strengths up to 2 per cent. inhibits far less than gelatin in 10 per cent. suspensions; and the difference in degree of inhibition exercised by 0.5 per cent. and 2 per cent. agar-agar is a small one.

Hæmolytic substances diffuse from gelatin into agar-agar more slowly than from saline into agar-agar. But the velocity of diffusion from agar-agar into gelatin is greater than from saline into gelatin.

The effects of differences in concentration of the hæmolytic agent vary according to the agent and the manner of its solution. When the hæmolyser is dissolved in salt solution the diffusion of 1:100 N. and 1:1000 N. solutions (saponin) is almost identical; while with solanin the stronger solutions diffuse faster. When the hæmolyser is dissolved in the colloid diffusion into fluid media is nearly proportional to the concentrations of the hæmolytic agent.

The velocity of diffusion into and from colloids is in general proportional to the square root of the time. Acids, alkalies, salts, and glucosides act in a manner which is in agreement with this rule. Cobra lysin and tetanolysin do not act in conformity with the rule.

Cobra lysin appears to diffuse into colloids more slowly, proportionally, than cobra neurotoxin, and tetanospasmin more slowly than tetanolysin.

The biological method described in this paper for studying diffusion in colloids is applicable to hæmolytic and some other toxic substances, and, with accuracy possibly only to such substances possessing relatively simple compositions.

Since all diffusion in the living body takes place within colloidal media of different concentrations, it would seem desirable to

perfect methods through which the interaction of toxic chemicals and the fluids and cells of the body may in a manner be imitated *in vitro*. Through this means our knowledge of toxicology may well be extended.



## THE CHEMISTRY OF ATHEROMA AND CALCIFICATION (AORTA).\*

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This investigation owes its inception to an attempt to study by micro-chemical methods the sequence of the changes occurring in the experimental lesions produced in the rabbit's aorta by the administration of adrenalin. As, however, micro-chemical methods proved unsatisfactory and analytical methods impossible, on account of the small amount of substance in the rabbit's aorta, the work as originally planned was abandoned in favor of a study by analytical methods of the chemistry of the various stages of arteriosclerosis in the aorta of man. The studies of Klotz,<sup>1</sup> which appeared shortly after the beginning of the work, and those of Wells,<sup>2</sup> which were published previous to its conclusion, are so recent that they render a general survey of the literature of calcification unnecessary.

As this work has to do mainly with the relation of the fatty acids and lecithin<sup>3</sup> (phosphorus) to calcification, the results of Klotz and Wells have a very important relation and the views of these investigators may be briefly stated at this time. Klotz employing chiefly micro-chemical methods believes he has demonstrated that the fatty acids play an important part in calcification. In a few qualitative examinations of calcified material, he found what appeared to be calcium soaps. These observations he attempts to support by micro-chemical studies which, however, are not convincing. His demonstration of soaps depends on a peculiar staining with Sudan III., of calcium — principally upon a black discoloration with an aqueous solution of silver nitrate (Kossa's stain). The occurrence in close contact of granules staining by each of these methods is taken as evidence

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of the presence of calcium soaps and the conclusion is drawn that the formation of these soaps is the first step in the process of calcification. The inaccuracy of this method of investigation has been pointed out by Wells, who, using purely analytical methods, reaches a different conclusion. He finds there is no essential difference between the processes of normal ossification and pathological calcification. No evidence was obtained to indicate that calcium soaps form a constant and important stage in the process of calcification. That they may be present in small amounts is not denied. His experimental studies also speak strongly against phosphoric acid, arising from decomposed nucleo-proteids, as an important binder of calcium.

The investigations here to be described deal with the study of nine aortas. Of these three were normal, two were aortas with extensive atheroma but no macroscopic evidence of calcification, and four showed various grades of calcification. One of these latter was the seat also of large "atheromatous ulcers." As several of the points studied have been covered by Wells' recent publication, these results will be stated but briefly.

The preparation of the material was as follows: The entire aorta from heart to division of the iliacs was removed and the intima and media carefully separated from the adventitia in order to exclude all extraneous fat. The material was now cut into small pieces, dried in absolute alcohol over a water bath, ground to a powder, and then placed in a flask with reflex condenser and boiled with absolute alcohol for periods varying from twenty-two to fifty-four hours and afterwards extracted in the Soxhlet apparatus for from nine to thirty-one hours. To facilitate extraction, the material was reground every twelve to fifteen hours and fresh alcohol added. The powdered residue was now extracted in boiling chloroform twenty-two to thirty-four hours and in the Soxhlet apparatus seven to fourteen hours. The use of ethyl ether,



which was one of the solvents first employed, had to be discontinued on account of the fact that numerous specimens reacted acid to phenolphthalein. Since this indicator served in the determination of the fatty acids, a neutral solvent became extremely essential. The extractive material taken up in absolute alcohol, boiling absolute alcohol, and chloroform was in each case entirely soluble in these solvents. An analysis of the extract was then made for calcium soaps, fatty acids, and lecithin as follows: To be absolutely certain of the solubility of calcium soaps, calcium stearate was prepared artificially. It was found to be sparingly soluble in cold absolute ethyl alcohol, considerably soluble in hot absolute ethyl alcohol, but insoluble in chloroform. According to Wells, "calcium oleate is less soluble in ether than in alcohol and the palmitate and stearate dissolve very little in ether, although considerably in ethyl alcohol; all dissolve much better in amyl alcohol, probably largely because of its high boiling point." According to the method of extraction, the calcium soaps should become soluble in the absolute alcohol and appear in the extractive material. The extracts were tested for calcium by fusing with sodium hydrate and potassium nitrate, precipitating with ammonium oxalate and weighing as  $\text{CaO}$ . The fatty acids were titrated in alcoholic solution with alcoholic  $\frac{\text{N}}{10}\text{NaOH}$  using phenolphthalein as indicator.

Since the phosphorus contained in the alcohol-chloroform extracts was wholly organic in character, it could be considered that the P-content depended upon the presence of some form of lecithin.<sup>4</sup> The  $\text{P}_2\text{O}_5$  content of the extracts was therefore determined by fusion, precipitation with molybdate solution, magnesium mixture and weighing as pyrophosphate and then calculated as distearyl lecithin. The remainder of the extractive material was estimated as neutral fats. The residue remaining after extraction was examined for Ca, Mg and  $\text{P}_2\text{O}_5$  by the ordinary analytical methods. In making comparisons with other work, however, we must emphasize the fact that the residue of the entire aorta was used and not merely isolated calcified areas.

RESULTS: I. Calcium soaps. — As far as we were able to determine no calcium soaps, or only mere traces, were to be found in normal or pathological aortas. This was definitely shown in the following manner. Extracts of the aortas which were completely soluble in absolute alcohol gave no reaction for calcium. Extracts, with supposed calcium content, dissolved in chloroform without leaving a trace of residue. As mentioned before, the soap — calcium stearate — is insoluble in chloroform.

II. Fatty acids and lecithin. — These will be considered together.

NORMAL AORTAS.

	Dried Substance.		Fatty Extractive.		
	Per Cent CaO.	Per Cent Fatty Extractive.	Per Cent Fatty Acid.	Per Cent Lecithin.	Per Cent Neutral Fat.
1 ..	1.4	20.3	6.1	18.3	75.6
2 ..	.3	11.9	5.73	16.3	77.97
3 ..	....	....	6.18	15.9	77.92

The surprisingly large percentage of lecithin is directly in line with the results obtained by Dunham<sup>5</sup> in his study of the normal kidney. He found that twenty-seven to thirty-seven per cent of the fatty extract was present in the form of lecithin.

Considering now the degree of calcification in the diseased aorta (the difference in calcification can be determined definitely by difference in calcium content of the residue), a striking relationship of the fatty acids, and especially of lecithin, to the grade of calcification is noticed. This is shown in the following table, in which the analyses are arranged according to the calcium content; the last two being those in which no calcification was evident macroscopically :

	Dried Substance.			Fatty Extractive.	
	Per Cent CaO.	Per Cent Fatty Extractive.	Per Cent Fatty Acid.	Per Cent Lecithin.	Per Cent Neutral Fat.
1 ..	11.9	22.5	9.6	16.6	73.8
2 ..	7.5	30.0	13.9	19.06	67.04
3 ..	7.2	22.4	19.09	24.2	56.7
4 ..	3.8	28.3	21.9	20.9	57.2
5 ..	1.7	8.1	20.2	37.9	41.9
6 ..	1.4	9.4	25.1	52.2	22.7

It is seen that the fatty acids and lecithin are present in greatest amount in the atheromatous aortas without gross evidence of calcification and that as the calcium content increases they rapidly diminish in amount. The remarkable increase in the amount of lecithin and fatty acids which appears at the initial stage of calcification certainly lends evidence to the fact of a decided "fatty" transformation occurring at this time. The fatty acids and lecithins here may or may not have a common origin. The parallelism in their appearance points to the probability that they do. The fact that lecithin and fatty acids may arise from a decomposition of substances of the "protagon" type, which Dunham has shown to exist in surprisingly large amounts in the normal cell, further substantiates this idea. The second stage in the fatty transformation consists in the disappearance of the lecithin and fatty acids coincident with the increasing calcium content of the tissue. The fatty acids probably react with the alkalis furnished by the blood and pass out of the cell as soluble compounds. The lecithin undergoes decomposition, liberating its phosphate radical, which serves either in part or entirely as the conjugant of the calcium.

III. Neutral fats. — The remainder of the fatty extractive was calculated as neutral fats. The results are shown in the above table. This procedure is not absolutely

correct, since necessarily numerous lipoids were present; for example, beautiful tests for cholesterol were obtained.

IV. The residue. — Unfortunately, it was impossible to examine only calcified areas for calcium, phosphates, and magnesium and at the same time to use all the material for fat analysis. Instead, the residue of the entire aorta was used. The entire  $P_2O_5$  estimated was considered as combining with  $MgO$  and  $CaO$  to form  $Mg_3(PO_4)_2$  and  $Ca_3(PO_4)_2$ . The remaining  $CaO$  was estimated as  $CaCO_3$ . In the aortas of lower calcium content there was relatively a small quantity of  $Ca_3(PO_4)_2$  while, as calcification proceeded, the proportion of calcium phosphate to carbonate increased. For instance, in an aorta with estimated 3.8 per cent  $Ca$  content, the relationship between the  $Ca_3(PO_4)_2$  and  $CaCO_3$  was as 69.5 to 25; that with 7.5 per cent  $Ca$  content as 74.7 to 24.8; and that with 11.9 per cent  $Ca$  content as 79.9 to 19.9.

As the calcification proceeded, however, the magnesium content decreased, being present in the more calcified areas as 0.1 per cent and 0.32 per cent.

Comparing our results with those of Klotz and of Wells,

1. They differ from those of Klotz, in that by analytical methods we were unable to find the calcium soaps which he claims to have demonstrated by micro-chemical examination, but are in accord with Wells' results in this regard.

2. The inorganic analyses of the residues differ, however, from those of Wells. Possibly this is due to the fact that the entire aorta was used. Such material necessarily included both calcified and normal areas, thus diminishing the absolute percentage of calcification. Again Wells examined a different kind of tissue (lymph node) and it is not impossible that the somewhat higher  $P_2O_5$  content obtained by him is due to the presence of the phosphorus containing proteids or their decomposition products, which are known to be present in such tissue in relatively large amounts. The  $Ca_3(PO_4)_2$  percentage was lower and the percentage of  $Mg_3(PO_4)_2$  much less. The figures do not

show the very close relationship to normal ossification which is so evident in his results.

CONCLUSIONS. — 1. The absence of calcium soaps in the extracts of all aortas examined leads us to the conclusion that in pathological calcification of the aorta at least, the formation of calcium soap is not an intermediate process.

2. The analyses of the residues apparently point to the fact, insisted upon by Wells, that in pathological calcification the inorganic salts are deposited in approximately the same proportion as in normal ossification. This does not necessarily signify, however, that the initial processes from which pathological calcification and normal ossification result are identical.

3. The remarkably high percentage of lecithin in the initial stage of calcification and the fact that with increasing calcium content there occurs a coincident diminution in the percentage of lecithin has led us to suspect that the phosphate radical may be supplied from a decomposition of lecithin.

[I wish here to express my indebtedness to Dr. Holmes C. Jackson, Director of the Laboratories of Physiological Chemistry and Experimental Physiology of the Albany Medical College, without whose instruction and supervision this investigation would have been impossible.]

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THE RESISTANCE OF THE RED BLOOD CORPUSCLES OF THE HORSE TO SALT SOLUTIONS OF DIFFERENT TONICITIES BEFORE AND AFTER REPEATED WITHDRAWALS OF BLOOD.\*

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(*Aided by the Rockefeller Institute for Medical Research.*)

In endeavoring to trace the causes leading to certain pathological states in horses used in the production of diphtheria antitoxin and repeatedly bled, one of us found a decided variation from horse to horse in the resistance of the red corpuscles to salt solutions of varying tonicity.‡ When the results were brought together they strongly pointed to repeated bleeding as the cause of lessened resistance, although none of the horses having corpuscles of less than average resistance had been followed from the start. It was also shown that corpuscles of lessened resistance to salt solutions were dissolved more readily in dilutions of their own serum as well as in that of other horses. This fact indicated that the tension of the serum of horses having corpuscles of less than average resistance does not necessarily rise to compensate for this lessened resistance.

The inference that lessened resistance was somehow associated with repeated bleeding was based on the fact that of the seventeen horses examined those most frequently bled possessed blood cells of least resistance. There were, however, among these several exceptions. A mare (No. 70) had corpuscles of relatively low resistance before any blood had been drawn and several horses which had been bled many times still maintained their corpuscles at a level nearly normal. It thus became necessary to analyze this variation

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\*Received for publication Nov. 1, 1906.

† During the past year very material assistance in the blood examinations was given by Dr. Paul A. Lewis.

‡ Theobald Smith; The pathological effects of periodical losses of blood. *Journal of Medical Research*, 1904, n.s. VII., p. 388.

more thoroughly and to determine in how far repeated losses of blood were actually responsible for a low resistance of the blood cells. We have therefore continued the work as outlined in the preceding paper and made large numbers of observations during the past two years, the results of which we shall very briefly discuss in the following pages. Three lines of observation were pursued: 1. About twenty additional horses were examined at different intervals to determine the level of resistance of their corpuscles. 2. A repeatedly bled horse with corpuscles of low resistance was examined from time to time during a long interval of rest to see whether any rise occurred in the resistance of the red cells. 3. Specially selected horses were bled repeatedly and at short intervals.

METHOD.—The procedure described in the preceding paper was followed, with but slight modifications, and the reader is referred to that paper to prevent repetitions. The salt solutions used ranged from .40 to .68 per cent. Great care was exercised in preparing successive solutions uniformly. The salt used for the stock solution was thoroughly ground and dried in a hot air oven at 130° to 140° C. for several hours, and the various dilutions made with the greatest care. We have usually prepared one hundred cubic centimeters of each dilution and used the same a number of times at intervals of a week or more. As a rule a stock solution of one per cent was made from which the various dilutions from .40 to .68 were then prepared. These were kept in old, glass-stoppered, frequently washed bottles, and evaporation avoided as much as possible. Comparative tests were made on the same corpuscles with old and new solutions whenever a new solution was first used. For other details the former paper should be consulted. In the estimation of the percentage tints, difficulties were encountered when the hemolysis was over sixty per cent. To rectify this difficulty two methods were employed. One was to add but one instead of two drops of corpuscles to those tubes in which hemolysis might exceed sixty per cent. The other



was to dilute the liquid with two or more volumes of water before estimating the tint.\*

A comparison of old and freshly prepared solutions indicated an increase in the tension of the former of from .00 to .02 per cent salt according to age, and relation of volume of fluid to the size of the bottle, that is to say, according to the opportunity for evaporation.

Among those who have studied the resistance of red corpuscles it has been customary to determine two limits, one represented by that strength of salt solution in which the red corpuscles just begin to lose hemoglobin, the other by that strength in which all or nearly all red cells are dissolved. These two limits are known as the minimum and the maximum resistance.

Although this procedure may be very useful in clinical work when very little blood is at the service of the experimenter, it was not considered sufficient for our purposes and we have, as in the earlier work, tested the resistance of the red cells towards intermediate grades of salt solution, using those .02 per cent apart. This gives a much more complete picture of the resistance and enables us to divide the corpuscles into as many classes or groups as there are salt solutions used. For example, in a salt solution of .62 per cent the corpuscles may begin to lose hemoglobin, as is shown by a tint of one-half per cent, in five hours. In a solution of .60 per cent the tint may be one per cent, in a solution of .58 per cent, two per cent. On the basis of these figures we may assume that about one-half per cent of all red corpuscles are destroyed in .62 to .64 per cent solution, one-half per cent in .60 to .62 per cent, and one per cent in .58 to .60 per cent and so on. A still finer division may be made by using solutions only .01 per cent apart, but we have considered this inexpedient owing to the amount of work involved.

#### VARYING RESISTANCE OF THE RED CORPUSCLES OF HORSES TO SALT SOLUTIONS.—If the hemolysis due to different

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\*The first method introduced an error in that a drop of blood corpuscles has a greater tension than the salt solutions used, but this error is so slight that we have not considered it.

percentages of salt solutions be plotted by laying out the latter as abscissæ and the percentages of hemolysis as ordinates a curve results. Illustrations of such curves from different horses will be found in Fig 1.

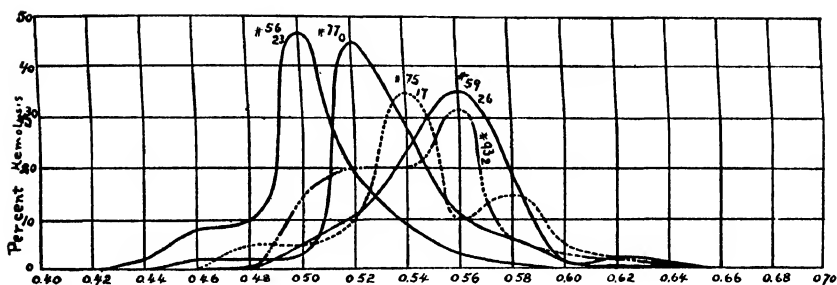


FIG. 1. — Five curves or frequency polygons showing the different groups of red corpuscles as differentiated by salt solutions at the end of five hours. The large number refers to the horse, the smaller number attached to it gives the total number of bleedings of five to six liters each.

The curves plotted from readings taken at the end of five hours teach us that the largest number of red corpuscles have a resistance nearly midway between the maximum and the minimum. Ranged on either side of this mean resistance are diminishing numbers having respectively less and greater resistance than the mean. The curve thus corresponds to a frequency polygon which in normal horses may be classed for the present as a simple unimodal nearly symmetrical curve with range limited in both directions.\*

The five selected curves in Fig. 1 illustrate a point already commented on in the preceding paper. The resistance of the corpuscles of horses — leaving aside for the moment the effect of bleeding — is not the same. We may divide horses into classes having corpuscles of maximum, of minimum, and of medium resistance. The extreme difference between those of maximum and those of minimum resistance we have found equivalent to .08 to .10 per cent salt solution, *i.e.*, to nearly one-half of the entire range of resistance of the horse's corpuscles.† Between these extremes all grades of

\* Davenport, Statistical Methods, page 19.

† In Fig. 1 the extreme difference is about .06 per cent.

resistance are encountered.\* The difference between individual horses is best brought out by comparing observations made on the same day and hence under identical conditions. This simultaneous comparison eliminates possible errors due to slight changes in the salt solutions used. The differences found by a direct comparison of series of tubes are recorded in the following table (I.) in form of an equation. The numbers refer to individual animals, the small figures to the number of bleedings of five to six liters each, and the decimal to the difference found in terms of sodium chloride per cent. Thus  $32_{17} = 54_{14} - .04$  means that horse No. 32 (47th bleeding) had corpuscles which were less resistant by about .04 per cent salt than those of No. 54 (14th bleeding); or, expressed in another form ( $54_{14} = 32_{17} + .04$ ), tubes containing corpuscles of No. 54 showed the same amount of hemolysis as those of No. 32, which contained .04 per cent more salt. In order to make the results clearer to the reader, each equation has been entered twice, that is to say once under each animal.

TABLE I.

$30_{59} = 68_6 - .01$	$54_{12} = 69_{25} - .03$
$30_{67} = 59_{20} + .04$	$54_{12} = 80_2 - .01$
	$54_{50} = 69_{33} - .05$
$32_{47} = 54_{14} - .04$	$54_{50} = 87_7 - .01 \text{ to } .04$
$32_{48} = 61_1 - .065$	$54_{50} = 93_6 + .015$
$32_{53} = 68_2 - .06$	
$32_{54} = 69_4 - .08$	$56_{14} = 59_8 + .06$
$32_{55} = 73_1 - .05$	$56_{18} = 72_6 + .02$
$32_{56} = 69_6 - .065$	$56_{23} = 68_{10}$
$32_{58} = 72_{13} - .045$	$56_{29} = 59_{23} + .06$
$32_{59} = 71_{38} - .065$	$56_{31} = 68_{18} + .025$
	$56_{32} = 54_{35} + .05$
$54_{14} = 32_{47} + .04$	$56_{40} = 89_5 + .01$
$54_{19} = 69_3 - .06$	$56_{40} = 75_{17} + .05$
$54_{20} = 59_{12} + .01$	$56_{49} = 87_8 + .01 \text{ to } .04$
$54_{21} = 66_6 - .04$	
$54_{33} = 75_1 - .02$	$59_5 = 72_4 - .05$
$54_{35} = 56_{32} - .05$	$59_8 = 56_{14} - .06$
$54_{41} = 69_{24} - .04$	$59_{11} = 65_3 - .07$

\* In the present paper we are not concerned with the rate of hemolysis in any given salt solution. This was discussed and graphically represented in the paper referred to.

TABLE I. — *Continued.*

$5912 = 5420 - .01$	$7211 = 7130 + .01$
$5913 = 705 - .015$	$7213 = 3258 + .045$
$5920 = 3087 - .04$	
$5922 = 6610 - .04$	$731 = 3255 + .05$
$5923 = 5629 - .06$	$735 = 700 + .02$
$5925 = 770 - .03$	$739 = 7014 + .02$
$5926 = 6617 - .04$	$7312 = 6818$
	$7312 = 771 - .005$
$611 = 3248 + .065$	
	$7416 = 6512 - .01$
$653 = 5911 + .07$	
$657 = 708 + .06$	$751 = 5433 + .02$
$6512 = 7416 + .01$	$750 = 5638 - .03$
	$7517 = 895 - .04$
$660 = 5421 + .04$	$7517 = 5640 - .05$
$6610 = 5922 + .04$	
$6617 = 5926 + .04$	$762 = 810 + .06$
$682 = 3253 + .06$	$770 = 5925 + .03$
$680 = 3059 + .01$	$771 = 7312 + .005$
$6810 = 5623$	
$6818 = 5631 - .025$	$781 = 800 - .03$
$6818 = 7312$	$781 = 791 + .01$
$693 = 5419 + .06$	$791 = 781 - .01$
$691 = 3251 + .08$	$791 = 800 - .03$
$696 = 3250 + .065$	$790 = 832 - .04$
$699 = 7130$	
$6914 = 7140$	$800 = 781 + .03$
$6924 = 5441 + .04$	$800 = 791 + .03$
$6925 = 5442 + .03$	$802 = 6925 - .02$
$6925 = 802 + .02$	$802 = 5442 + .01$
$6933 = 5450 + .05$	
$6933 = 877 + .05$	$810 = 762 - .06$
$6933 = 930 + .065$	$813 = 841 - .02$
$705 = 5913 + .015$	$8210 = 880 + .02$
$708 = 657 - .06$	$8210 = 932 + .06$
$709 = 735 - .02$	$8213 = 868 + .005$
$7011 = 739 - .02$	
	$832 = 790 + .04$
$7130 = 7211 - .01$	$838 = 851 + .015$
$7130 = 690$	$838 = 861 + .01$
$7138 = 3259 + .065$	
$7140 = 6914$	$841 = 813 + .02$
	$848 = 857 + .015$
$721 = 595 + .05$	
$720 = 5618 - .02$	$851 = 838 - .015$

TABLE I. — *Concluded.*

$85_1 = 86_1 + .005$	$89_5 = 56_{46} - .01$
$85_7 = 84_8 - .015$	
$85_7 = 93_3 + .06$	$90_3 = 91_1 - .05$
$86_1 = 83_3 - .01$	$91_1 = 90_3 + .05$
$86_1 = 85_1 - .005$	
$86_8 = 88_7 + .02$	$93_2 = 82_{10} - .06$
$86_8 = 82_{13} - .005$	$93_2 = 88_6 - .04$
	$93_3 = 85_7 - .06$
$87_7 = 54_{50} + .02 \text{ to } .04$	$93_6 = 54_{50} - .015$
$87_7 = 93_6 + .01 \text{ to } .06$	$93_6 = 69_{33} - .065$
$87_8 = 56_{10} - .01 \text{ to } .04$	$93_6 = 87_7 - .01 \text{ to } .06$
$87_7 = 69_{33} - .05$	$93_9 = 94_1 - .04$
	$93_9 = 95_1 - .08$
$88_6 = 93_2 + .04$	
$88_6 = 82_{10} - .02$	$94_1 = 93_9 + .04$
$88_7 = 86_8 - .02$	$94_4 = 95_1 - .04$
"	
$89_5 = 75_{17} + .04$	$95_1 = 94_1 + .04$
	$95_1 = 93_9 + .08$

A glance at this table shows that horses Nos. 32, 59, 93, 54, 70 and 75 have corpuscles of lower than average resistance. Certain others, such as Nos. 76 and 65, have a resistance above the average.

It must not be assumed, however, that the difference in hemolysis between series of tubes of two horses is necessarily the same from .40 to .70 per cent as might be inferred from the general equation of the table. As a rule, the difference is greater in the lower percentages and grows smaller towards the higher. In some instances it is very uniform throughout, rarely, as in No. 87, it is irregular. The differences given in the table represent most accurately those observed in the middle of the series of salt solutions. The actual differences which cannot be expressed in form of a simple equation are best studied by an examination of the curves in Figs. 1 and 3.

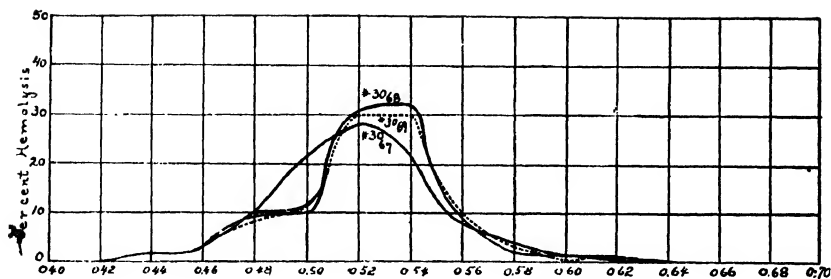


FIG. 2.—Three curves plotted at three successive bleedings one week apart.

THE RELATION OF REPEATED BLEEDING TO CHANGES IN THE RESISTANCE OF RED CORPUSCLES. — In the former paper, as already stated, the preliminary inference was drawn that low resistance was due to repeated bleeding. Additional data on this question have been collected during the past two years. These are too voluminous to present in any detail and we content ourselves with the following brief synopsis of the experiments:

*α.* Special cases: No. 32. — In the preceding paper the low resistance of the corpuscles of this horse was described and illustrated by curves. If this condition was due to abstraction of blood, it was reasonable to suppose that a rise in resistance might be anticipated when bleeding was stopped. On October twenty-ninth, 1903, the injection of diphtheria toxin was stopped, but the bleeding continued, at the rate of five and one-half liters every twenty-five days. After March fourth, 1904, only small amounts of blood were drawn for counts and resistance tests. Up to October thirty-first no change in the resistance of the red cells beyond that within the margin of error of the methods employed could be detected.

To determine whether successive withdrawals of blood one week or two weeks apart would have any effect upon the resistance of the red cells, a horse (No. 30) was taken which had been bled sixty-six times since 1900 and whose corpuscles had maintained the average resistance or were perhaps about

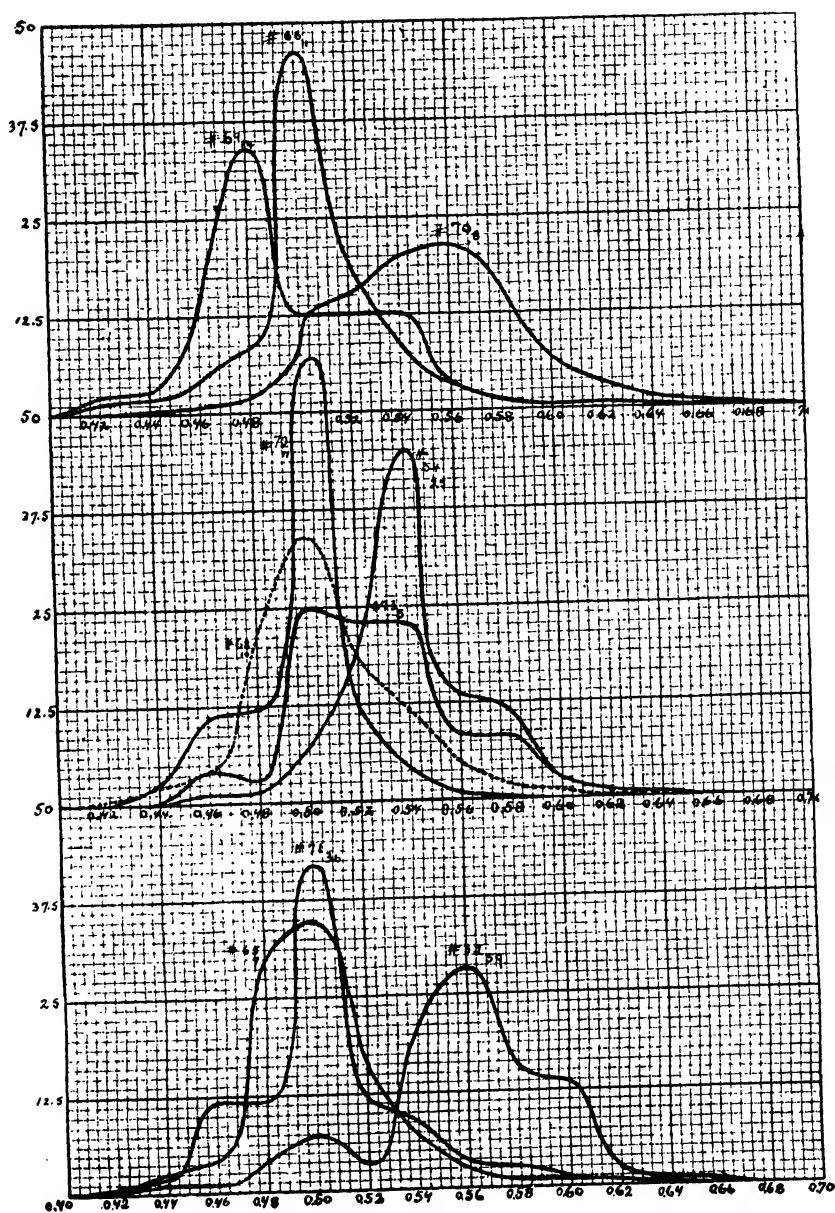


FIG. 3. — Curves from different horses showing the different groups of corpuscles after a twenty to twenty-four-hours' exposure to the salt solutions.

.02 per cent less resistant than the average. After two withdrawals of five and one-half liters within a week, no change in the resistance could be detected one week after the second withdrawal. The three consecutive resistance curves are plotted in Fig. 2. The subsequent history of this horse is given in the article by Dr. P. A. Lewis in this number, page 449.

In another horse (No. 59) whose corpuscles were of a low resistance, the abstraction of five liters of blood (on March 6, 20, and 27) did not make any appreciable impression on the resistance as determined on March 27, April 3, and April 21.

Horse No. 82, weighing eleven hundred pounds, was bled ten times between October fourteenth, 1905, and May third, 1906. Five liters were withdrawn each time. The resistance of the red corpuscles at the tenth withdrawal was about the same as at the beginning.

Horse No. 93 was used as a control. Black mare, eight to nine years old, weighing nine hundred and twenty pounds. Repeatedly bled at intervals of seven to ten days. Received no toxins at any time. The figures in Table I. show that the red cells of this horse had a resistance well below the average from the start.\* Between April twenty-seventh and June twenty-fifth, 1906, this horse was bled nine times, five liters being withdrawn each time. A comparison of the protocols of the resistance tests made at the second and the ninth bleeding indicates an average lessened resistance of .015 to .02 per cent salt. This is very slight when we bear in mind that thirty-five liters of blood had been withdrawn between the two tests in a period of about seven weeks. If we allow about seventy pounds of blood for the horse, about eighty pounds, or more than the entire blood in the body at any given time, had been removed in the short period of seven and one-half weeks. There was, however, more or less disturbance in the individual groups of corpuscles as shown in Fig. 4.

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\* See also curves in Figs. 1 and 4.



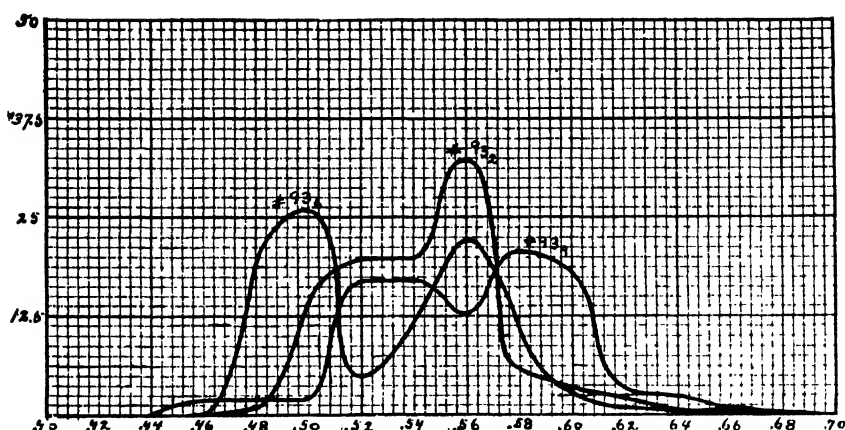


FIG. 4. (See p. 443.)

Horse No. 56, chestnut gelding, about nine years old in 1902. Weight eleven hundred and twenty-seven pounds at that time. This horse was bled fifty times from November, 1902, to July, 1906. The resistance of the red corpuscles, examined first in November, 1903, was found to be normal. Five and one-half liters of blood were drawn every three and one-half to four weeks, with the exception of one period of about six months when the amount drawn was eight liters at a time. A careful comparison of the early records and the latest does not reveal any appreciable fall in the resistance of the red cells.

*b. Miscellaneous cases.* — Since the preceding paper was written, about twenty additional horses have been studied, of which two (Nos. 82 and 93) have been specially referred to in the preceding pages. All of these have been followed from the beginning. In one (No. 93) the resistance was found at the start nearly as low as in any of the earlier horses. In two others a medium degree of subnormal resistance was found (Nos. 75 and 90).

In none did any marked fall in resistance occur as a result of the usual abstraction of blood, during periods of one to three years. The maximum fall noted has been .02 to .025 per cent salt. Thus in No. 75 this fall was noticed after

seventeen bleedings extending over a period of fifteen months. No. 68 showed the same decline in eight bleedings. In other cases the change was not appreciable. Even a change of .02 per cent may fall within the margin of error of the method employed.

These different kinds of data all point to the inference that the extreme differences between certain horses in the resistance of the red corpuscles cannot be accounted for by loss of blood alone. On the contrary, the evidence thus far brought together attributes but a little to loss of blood. The chief phenomenon appears to be individual variation due to still unknown causes just as the capacity to yield diphtheria antitoxin is.\*

In no instance have we observed enough change in the resistance of corpuscles to take place during the period of bleeding to permit us to classify an animal of originally normal resistance as one of low resistance. In other words, we have not been able to reproduce those differences by bleeding which we have encountered spontaneously.

THE USE OF SERUM DILUTIONS TO DETERMINE THE RESISTANCE OF RED CORPUSCLES. THE USE OF RED CORPUSCLES TO DETERMINE THE OSMOTIC TENSION OF SERUM. †  
— In the preceding paper attention was called to the method of comparing the resistance of red corpuscles of horses in dilutions of their own serum as well as in that of other horses, and the conclusion was drawn that this method reveals equally well differences in the resistance of the red cells.

It was recently extended and perfected so that it may take the place of the salt-solution method, provided sufficient control tests are introduced to eliminate the effect of a possible variation in the tension of the serum itself. It is of

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\* Such individual differences are furthermore expressed in the depth of color of the serum (when free from all hemoglobin), the rapidity with which the clotted blood contracts and with which corpuscles settle down in defibrinated blood. Another more significant variation occurs in the amount of the corpuscular deposit in defibrinated blood. It would be of interest to ascertain whether these variable characters persisting in the individual are transmitted to succeeding generations.

† Hamburger. Osmot Druck, I. S., 359.

course inapplicable where the amount of blood obtainable is small.

In the carrying out of this procedure, the same details should be followed from test to test to ensure uniformity of results. The standard tints prepared were fifty, forty, thirty, twenty-five, ten, five, and two per cent. By taking the standard amount of sedimented corpuscles in three cubic centimeters of water and adding definite amounts of this to serum and water, the above standard tints are easily prepared. It is necessary to use serum because of the color imparted by it. Thus for the above percentages the following dilutions were made with a laked fluid made up in the proportion of two drops of blood corpuscles to three cubic centimeters of water containing a trifle salt to avoid cloudings :

Serum .....	2	2	2	2	2	2	2	2
Water .....	0.0	0.0	0.1	0.4	0.55	0.7	0.85	0.94
Laked blood .....	2.0	1.33	0.9	0.6	0.45	0.3	0.15	0.06
Per cent of tint.....	50	40	30	20	15	10	5	2

It is also desirable to have control tubes of the undiluted and diluted serum, for the serum of horses may differ appreciably in depth of color. Some are pale, watery, others a deep golden yellow. This impresses itself upon the control tints.

As in the tests with salt solutions, small test-tubes were used and definite quantities of serum and distilled water amounting in each case to three cubic centimeters were added to each tube. After shaking the tubes thoroughly one or two drops of sedimented horse corpuscles were added to each tube. This was again shaken up to suspend the corpuscles uniformly. The tubes were kept at room temperature for about five hours, then the tints carefully measured by comparison with a series of controls made with laked blood as described above and the tubes gently shaken again and placed over night in a temperature of 10° to 15° C.

Next morning at the end of twenty to twenty-four hours a final reading was made.

A difficulty arises in the exact estimation of the percentage tint when the hemolysis is forty per cent or above. As this happens frequently with the corpuscles of certain horses it became necessary to meet it. One method was to dilute portions of tubes in which hemolysis was over forty per cent with one or two volumes of water. This was not satisfactory unless serum was used for dilution. A method more economical of time and serum than this is the one referred to above — to add less blood corpuscles — one drop in place of two — to those tubes in which hemolysis may be high and multiplying the resulting tint by two.

The following table illustrates the general method of procedure, and shows at the same time the pronounced differences between the corpuscles of certain horses :

TABLE II.

Serum dilution.			I.	II.	III.	IV.	V.	VI.	VII.
		Serum of	No. 93.	No. 95.	No. 93.	No. 94.	No. 94.	No. 95.	No. 93.
Serum + water, cc.	Per cent serum.	Corpuscles of	No. 93.	No. 93.	No. 91.	No. 94.	No. 93.	No. 95.	No. 95.
1.7 + 1.3	56½	Per cent hemolysis after 24 hours.	100	80	80	80	100	10	30
1.8 + 1.2	60		90	60	50	40	85	6	12
1.9 + 1.1	63½		70	11	15	12	60	Trace.	5
2.0 + 1.0	66½		30	5	5	3-4	30	0	Trace.
2.1 + 0.9	70		10	1-2	Trace.	Trace.	8	0	0
2.2 + 0.8	73½		3	?	0	0	3	0	0
3.0 + 0.0			0	0	0	0	0	0	0

From this table we may obtain information about the resistance of the red corpuscles as well as the osmotic tension of the serum.

Comparing columns I. and II., which show the percentage hemolysis of corpuscles of 93 in serum of 93 and 95, we find that the osmotic tension of serum 95 is four to five per cent serum higher than 93. Comparing columns VI. and VII. we

see again that the osmotic tension of serum 95 is about four per cent serum higher than 93. Comparing columns I. and V., and columns III. and IV. we learn that the osmotic tension of the serum of 93 and 94 is practically the same. It will be seen that this determination is made by adding corpuscles of the same horse to the sera of different horses.

Turning now to the resistance of the corpuscles, we may determine this from the table by comparing the hemolysis of the corpuscles of different horses placed in the same serum. Thus, comparing columns I. and III. and columns IV. and V. we learn that the difference in resistance between the corpuscles of 93 and 94 is four to four and five-tenths per cent serum. Knowing that the tension of serum 93 and 94 is the same, we conclude that corpuscles 93 are less resistant than those of 94 by four to four and five-tenths per cent serum.

Comparing the resistance of corpuscles of Nos. 93 and 95 (columns II. and VI., and I. and VII.), we find that the difference in the first case is seven per cent serum, in the second ten per cent in favor of No. 95. This slight discrepancy cannot at present be explained. It seems to indicate that after eliminating the difference in serum tension the corpuscles of 93 are laked more easily in their own serum than in that of 95.\*

Finally, comparing columns III. and VII. the difference between corpuscles of 94 and 95 is about four per cent serum. Thus we obtain the independent equations:

- (1) Corpuscles of  $93_0 =$  corpuscles of  $94_1 - 4$  to 4.5 per cent serum.
- (2) " "  $95_1 =$  " "  $93_0 + \frac{1}{4} (10 + 7) = 8.5$  per cent serum.
- (3) " "  $95_1 =$  " "  $94_1 + \frac{1}{4}$  per cent serum.

From (1) and (3) we also get:

- (4)  $95_1 = 93_9 + 8$  to 8.5 per cent, which is identical with (2).

Comparing the results obtained with salt solutions at the same time we have the following close agreement:

939 = 941 - .04 per cent salt solution.

$$939 = 951 - .08 \quad " \quad " \quad " \quad "$$

$$95_1 = 94_1 + .04 \quad " \quad " \quad " \quad "$$

\* This has been observed in another case, though the difference amounted to but one per cent serum.

We note that the percent of serum is nearly the same as the hundredths per cent salt in these equations. This is due to the fact that the osmotic tension of serum is equivalent to about 0.9 per cent salt solution. This figure is obtained by comparing the per cent hemolysis in the salt solutions and in the serum dilutions. Thus if the laking effect of sixty per cent serum is the same as that of 0.53 per cent salt solution, the tension of undiluted serum would be  $\frac{.53 \times 100}{60} = .88$  per cent salt solution.

In the same manner the following data concerning serum tension and corpuscle resistance were determined. These it will be noted agree very closely with the data obtained with simple salt solutions.

TABLE III.

Comparison of the Serum Tension of Two Horses in Per Cent of Serum.	Resistance of the Corpuscles of Two Horses Compared. The Difference Expressed in Per Cent of Serum.
$56_{46} = 75_{17} + 5\%$	$56_{46} = 75_{17} + 6\%$
$56_{46} = 80_5$	$56_{46} = 89_5$
$59_8 = 63_2$	$69_6 = 32_{56} + 6\frac{2}{3}\%$
$69_6 = 32_{56}$	$69_{33} = 54_{50} + 4 \text{ to } 5\%$
$69_{33} = 54_{50}$	$69_{33} = 87_7 + 4 \text{ to } 5\%$
$69_{33} = 87_7 - 4 \text{ to } 5\%$	$75_{17} = 89_5 - 5\%$
$79_6 = 83_2 - 3\frac{1}{3}\%$	$79_6 = 83_2 - 4\%$
$80_1 = 78 \cdot + 1\%$	$82_{10} = 86_7 + 1\frac{2}{3}\%$
$82_{10} = 86_7$	$82_{10} = 88_6 + 4\%$
$85_7 = 84_8 + 1\frac{2}{3}\%$	$85_7 = 84_8 - 2 \text{ to } 3\%$
$86_7 = 93_6 + 1\frac{2}{3}\%$	$86_7 = 88_6 - 3\frac{1}{3}\%$
$88_6 = 86_7 + 1\frac{2}{3}\%$	$93_9 = 94_1 - 4 \text{ to } 4.5\%$
$93_9 = 94_1$	$95_1 = 94_1 + 4.5\%$
$93_9 = 95_1 - 4\%$	$95_1 = 93_9 + 8\%$

As stated in the preceding paper, serum tension and resistance of red cells are not parallel to each other. In the table we find horses having corpuscles of low resistance whose serum tension is low (No. 75), normal (Nos. 32, 54), and high (No. 87).

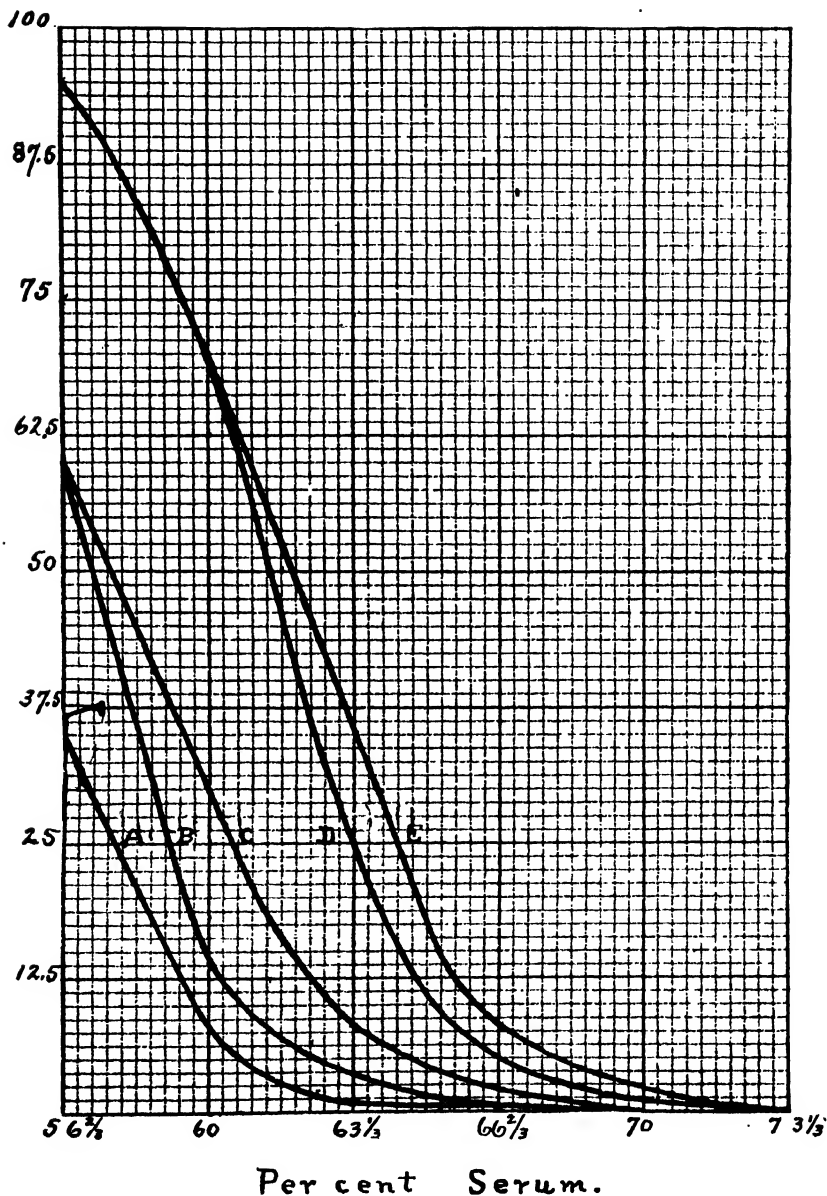


FIG. 5. — Curves constructed by plotting the total hemolysis on coördinates over the serum dilutions on the axis of abscissæ. The serum used in all cases is that of horse No. 86. The corpuscles tested are from No. 82 (A), 86 (B), 88 (C), 93 (D, E). The tests recorded by B, C, D were made twenty-four days later than those of A and E. It will be noticed that corpuscles of No. 82 are the most resistant, those of No. 93 the least.

Our records are not complete enough to enable us to state whether this serum tension is, like the resistance of the red cells, a fairly constant individual factor or one varying from time to time. This would most probably be determined with greater accuracy by freezing-point determinations which eliminate the chance (which, however, is very slight indeed) of variations in the resistance of corpuscles of the same horse used to test the serum tension from time to time.

ISOLYSINS. — In the use of serum dilutions to determine both the resistance of red corpuscles and the osmotic tension of the serum, it was shown that by placing the corpuscles of the same horse into different sera, and the corpuscles of different horses into the same serum these objects could be attained and the errors resulting from different tonicity of sera eliminated. There is, however, one source of error recently encountered to which attention should be called. It is the existence of isolysins in a small number of horses, perhaps ten per cent. These had been sought for from the start, but none found, hence the statement in the former paper that isolysins were not encountered.

Two horses having in their serum lysins towards the corpuscles of other horses have been under observation. Though both these animals are producing antitoxin, it is assumed that the lysin is not the result of any toxin treatment. Lysis of red corpuscles of other horses up to fifteen per cent results in undiluted serum and the increased hemolysis over and above that due to dilution of the serum is evident throughout the series. Very recent tests indicate that it is heat-labile and disappears when the serum is heated at 55° C. for one-half hour. These isolysins will be studied more in detail and form the subject of a later communication. One object in referring to them here is to warn any future observers of the existence of these bodies and point out that they interfere with resistance tests in diluted serum.

#### GENERAL CONSIDERATIONS AND SUMMARY.

About ten per cent of the horses which have come under observation have red corpuscles of a resistance so low as



compared with the other ninety per cent that even crude methods could have detected it. The use of accurate methods carried out uniformly from month to month showed little if any change or fluctuation in this low resistance over a period of one to three years. The same results were observed when dilutions of horse serum were employed in place of salt solutions. As shown in Table III. the osmotic tension of the serum from various horses was the same with few exceptions, whereas the resistance of the red corpuscles was subject to considerable fluctuations, from case to case.

The influence of repeated bleedings has been shown to be relatively slight. In a few horses there was noticed a lowering of resistance equivalent to .02 to .025 per cent salt solution.

It is highly probable that with more severe losses of blood some impression can be made upon the inner form of the frequency polygon, which represents graphically the different groups of corpuscles. The only horse which we have subjected to any severe bleeding was No. 93. This animal has already been referred to. A plotting of three blood examinations is shown in Fig. 4. The earliest curve (93<sub>2</sub>) approximates the unimodal curve. The curves obtained from corpuscles at the sixth and ninth bleedings depart markedly from the normal and indicate a decided rearrangement of the various groups of corpuscles, resulting in bimodal curves. It would appear as if the severe drain brought into the circulation more corpuscles of a given group than occur normally in the blood. We may then assume, pending other trials of severe blood-letting within a short space of time, that the monthly losses of one-seventh of the total blood is well borne and does not appreciably change the form of the frequency polygon or curve of resistance as we have plotted it, but that severe losses may modify the curve without affecting appreciably the maximum and the minimum resistance.

Turning to some physiological aspects of the variation in resistance, we have to consider its relation to sex and age of the individual, and to age and size of the red cells themselves. Before doing this it may be well to enquire what the

normal resistance to salt solutions may be considered to be. Inasmuch as we have variates ranging themselves on either side of the mean, this mean may be called normal for purposes of standardization and comparison of results. A tabulation of the various results obtained leads us to establish two points for the horse which can be used for comparison. (1), The total hemolysis in .50 per cent salt solution, and (2), the salt solution corresponding to fifty per cent hemolysis. For (1) the mean or normal is thirty to forty per cent hemolysis. For (2) the mean or normal is .49 per cent salt solution. Or, stated in round numbers, in the average horse .50 per cent salt causes a total of fifty per cent hemolysis, and hence fifty per cent hemolysis is caused by .50 per cent salt solution.

The following table exemplifies the rule and readily reveals the horses with corpuscles of low resistance:

TABLE IV.

Number of Horse.	Per Cent Salt Solution Corresponding to 50% Hemolysis.	Per Cent Hemolysis Corresponding to .50% Salt Solution.
30 <sub>61</sub>	.505	60
32 <sub>59</sub>	.546	85
54 <sub>25</sub>	.529	90
56 <sub>23</sub>	.492	33
59 <sub>16</sub>	.554	95
69 <sub>9</sub>	.491	33
70 <sub>8</sub>	.535	80
75 <sub>17</sub>	.537	90
84	.493	35
85 <sub>1</sub>	.492	38
91 <sub>1</sub>	.492	38
93 <sub>9</sub>	.552	92
94 <sub>1</sub>	.51	64
95 <sub>1</sub>	.473	25

The relation of sex to resistance of red corpuscles is quite striking. Of thirty-eight horses examined during the past three years, twenty-one were males and seventeen females. Of the six with corpuscles of low resistance all were females.\* In age all the horses examined ranged from eight to possibly twenty years. The same differences in age are found among those with corpuscles of low resistance.

This low resistance may be a phenomenon of early life which in some individuals does not change as the animal becomes adult. We have not been able to examine young horses to clear up this point. If it should prove to be characteristic of the young, its persistence in later life may be a racial character evolved by breeding. It is not peculiar to either well-bred or ordinary breeds, for we have been able to examine both kinds and we do not find it belonging to one or the other class.

Turning now to the red corpuscles themselves we may ask whether there is any relation between resistance to salt solutions and age. This is a purely physiological question, but it seems desirable to point out certain inferences which may be drawn from our data on this point. Hamburgert† assumes that there is such relationship, but does not define it. Under this assumption there are two possible theories. First, the youngest corpuscles may be least resistant; second, the oldest corpuscles may be least resistant.

Assuming that the youngest corpuscles are the most resistant and that the oldest are the least resistant and the ones destroyed, we would have for our polygon not the one we have figured but one rectangular in outline. If the youngest corpuscles are the least resistant to salt solutions and oldest corpuscles the most resistant, but still the ones to be destroyed, the polygon would also be rectangular. If the youngest corpuscles were the most or the least resistant and if the destruction were haphazard and liable to invade

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\* It should also be stated here that the two horses whose serum contained isolysins were females. One of them had corpuscles of normal resistance, the corpuscles of the other were below normal.

† Osmot Druck, I., p. 363.

any group of cells the curve would be irregular and dependent upon accidents.

Neither of these hypotheses accords with the facts. The theory most in harmony with them disavows any relationship between age of corpuscles and resistance to salt solutions, and assumes that the different groups are produced and destroyed independently of their resistance to salt solutions. The difference in the various groups may perhaps be due to their different origin, some coming from the marrow of the ribs, others from that of the head of the femur, still others from the shaft of the same bone and so on. This theory might account for the abnormal curves in No. 93 (see Fig. 4). Places in the osseous system of this horse hitherto called upon but slightly may have furnished large numbers to meet the severe drain.

The possible relation between diameter of corpuscles and their relative resistance to salt solutions was taken into consideration. A large number of measurements were made, but the results did not show any close correspondence between size and resistance. Nevertheless we do not wish to convey the impression that our work is in any way final. Here, also, more repeated and larger bleedings may be necessary to bring about such exaggerated conditions as are suitable for a further study of this problem.

Turning finally to the pathological significance, the question naturally arises, is the animal with corpuscles of low resistance more prone to certain diseases than the one with corpuscles of normal resistance? The data collected during the years of observation indicate that the former are no more predisposed than others to the ill effects of toxin injections and repeated bleeding. This is brought out in the following paper by Dr. P. A. Lewis. Another disease to which the horse is subject is azoturia or hemoglobinuria. This is characterized by the presence of hemoglobin in the urine. Large draught horses are most susceptible and the disease occurs chiefly in winter.

Among our animals there were five which had wholly or partially recovered from azoturia. Of these only one (No.

32) had corpuscles of low resistance. This showing is hardly sufficient to associate azoturia with such corpuscles.

The relation of low resistance to any increased susceptibility to specific hemolysins has not been investigated.

Our results cannot be generalized to other species, but these must be studied in the same laborious way to determine to what extent they are influenced by losses of blood and how far individual, hereditary or developmental characters enter to cause original differences in the resistance.

Attention should be drawn, however, to the uncertainty now surrounding the resistance tests of human corpuscles hitherto recorded. Two facts stand out quite preëminently in our results, individual variation and marked stability of this variation. In the human subject statements of lowered resistance cannot be fully accepted until individual and racial variations in health have been studied. If such variations are found to occur then the resistance formula for any diseased condition must be preceded by the normal formula for that individual, for it may occur that, as in the horse, the individual, normal variation is greater than any attributable to disease.

On the whole, it may be said that the study of the resistance of red corpuscles to different osmotic tensions does not appear to have much pathological significance if such extreme differences as those we have found in horses are compatible with health and repeated losses of blood. The subject nevertheless merits more attention on the part of clinical medicine. Statistical observations among human beings living in both temperate and tropical climates are especially to be desired. It is in the tropics where the osmotic tension of the serum may be subjected to far greater fluctuations than in the temperate zones. There, also, a high degree of immunity toward intraglobular parasites exists.



## HEMORRHAGIC HEPATITIS IN ANTITOXIN HORSES.\*

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and the*

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*(Aided by the Rockefeller Institute for Medical Research.)*

About six years ago the attention of the director of these laboratories was called to unusual developments in certain horses used to produce diphtheria antitoxin. Some horses after years of successful use would die suddenly, post-mortem examination revealing, as the immediate cause of death, extensive hemorrhage into the peritoneal cavity from a ruptured liver. Because of its practical importance to those engaged in the production of antitoxins and its possible theoretical significance, this subject was thought worthy of the most careful investigation in its diverse aspects. The earliest work on the subject seemed to indicate that pathological changes in the red blood corpuscles might be the basis of the disease. Experiments primarily conducted with a view to deciding whether or not this were true have been carried to completion and the results have been published.† They show that the erythrocytes are but little altered in their resistance to hemolysis in hypotonic sodium chlorid solutions by immunization and repeated bleeding, and that the osmotic tension of the serum is not essentially changed.

The work which forms the basis for this paper consisted in a study of the records of the treatment, and a careful gross and microscopic post-mortem examination of such horses as died or were killed. In some instances the usual routine treatment for the production of antitoxin has been modified by an increase in the frequency of bleeding. One horse not treated with diphtheria toxin and one treated for a short time only have been bled repeatedly, killed by shooting and studied anatomically.

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\* Received for publication Nov. 4, 1906.

† See preceding paper. — *Ed.*

This work shows that prolonged treatment of the horse with diphtheria toxin, bleeding repeatedly during the same period, causes amyloid degeneration of the liver and, less often, of the spleen. After amyloid degeneration of the liver has developed, increasing the frequency of the bleeding, while keeping the quantity of blood drawn each time, a constant, brings about hemorrhage into the organ, which may rupture through the capsule and terminate fatally. In its earliest stage the hemorrhagic lesion is an infarct produced by the obstruction to the circulation through the sinusoids of the peripheral portions of the liver lobule.

The preliminary work on this condition was done by Dr. Theobald Smith and he has been actively interested in its completion. I thank him sincerely for the opportunity he has afforded me for an interesting and profitable study.

While this disease of the horse is essentially an artificial or experimental one, taking its origin in the new service into which the animal has been pressed in recent years, it is none the less, from the point of view of morbid anatomy, closely allied to if not identical with, conditions that have long been known to veterinarians. The standard text-books on the pathological anatomy of domesticated animals have short chapters devoted to amyloid liver and rupture of the liver in the horse, and the relation of the one condition to the other is usually commented on. Under these titles Friedberger and Frohner in the appendix to the latest edition of their text-book give a complete index to the literature of these subjects referring to the diseases arising under natural conditions. Their list includes seventy titles.

It is probable that these diseases of the horse are much more common in Europe than in this country. Nevertheless the possibility of encountering a naturally diseased animal would have to be borne in mind constantly while conducting experiments along these lines and any too striking result may have to be criticised with such a coincidence in mind. Whether the very definite series of anatomical changes which our cases present is constant in the apparently similar cases arising under natural conditions is perhaps doubtful.



In a discussion on amyloid degeneration at the meeting of the Deutsche Pathologische Gesellschaft in 1904 this condition was considered in part, and in its present relationship. In this discussion Zenoni was credited with observing amyloid in the livers of antitoxin horses and Kraus considered that the observation was especially important, as it might afford an opportunity for comparing amyloid degeneration artificially produced with that arising in the same species of animal under natural conditions.

At the recent meeting of the American Association of Pathologists and Bacteriologists (Baltimore, 1906), Pearce and Pease gave a preliminary report of pathological lesions in antitoxin horses. Their paper has not to our knowledge been published. A communication they kindly sent us states that amyloid degeneration has been present in but two of their cases and that thrombosis of the veins has been an important feature in most of them.\*

Our material comprises eleven horses. The accompanying table shows the chief facts concerning the horses, their treatment and the result.

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\*About three years ago the vice-director of the Serum Institute in Bern told Dr. Smith that some of their horses had died of hemorrhage from the liver. Nothing has to our knowledge been published from that laboratory.

TABLE I.  
*Summary of horses, treatment and results.*

Number of Horse.	Sex.	Age.	Time Under Treatment.	Cause of Death.	Treatment.	Found at Post-mortem Examination.
III. . . . .	Male.	12 years.	5 years.	Shot.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 54 times. Interval — 33d to 34th, 1 week.	Extreme amyloid degeneration of liver. Subcapsular hemorrhage several inches in diameter. Microscopic hemorrhages into centers of liver lobules. Dilatation and rupture of central veins.
XVII. . . . .	Female.	. . . . .	4 years.	Rupture of liver hemorrhage.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 47 times. No increase in frequency. Died in morning after last bleeding.	Extreme amyloid degeneration of liver. Large recent hemorrhages into liver. Rupture of liver. Hemorrhage into peritoneal cavity. Old encysted hemorrhages of liver.
XXX. . . . .	Male.	12 — years.	5½ years.	Rupture of liver hemorrhage.	Diphtheria toxin. Maximum 500 cc. periodically. Bled 71 times. Intervals: — 67th to 68th, 1 week } 68th to 69th, 1 week } 69th to 70th, 1 week } 70th to 71st, 1 week } Died one month after last bleeding.	Extreme amyloid degeneration of liver. Recent large hemorrhages. Rupture of liver. Hemorrhage into peritoneal cavity. Old partly organized hemorrhages of liver. Regeneration of liver parenchyma.
XXXII. . . . .	Female.	11 years.	4½ years.	Shot.	Diphtheria toxin. Maximum 600 cc. Bled 39 times. Toxin stopped for one year. Bleeding stopped for eight months. Then bled twice with interval of one week. Killed one week later.	No amyloid degeneration. No hemorrhage.
LIX. . . . .	Female.	12 years.	2½ years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 25 times. Intervals — 22d to 23d, 2 weeks. 23d to 24th, 1 week. 24th to 25th, 1 week.	Trace of amyloid degeneration of liver. Well marked amyloid degeneration of spleen. Acute hemorrhagic nephritis.

LXXI. . . .	Male.	12 + years, 6 $\frac{1}{4}$ years.	Shot.	Typhoid toxin. Five years. 20-100 cc. Diphtheria toxin. 1 $\frac{1}{2}$ years. Maximum 500 cc. Bled 41 times. No increase in frequency of bleeding.	Well marked amyloid degeneration of liver. Extreme amyloid degeneration of spleen.
XI. . . . .	Male.	9 years.	Septicemia secondary to strangulated hernia.	Diphtheria toxin. Maximum 700 cc. Bled 13 times. No increase in frequency of bleeding.	No amyloid degeneration. No hemorrhage.
XXIII. . .	Male.	15 years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 48 times. No increase in frequency of bleeding.	No amyloid degeneration. No hemorrhage.
LVI. . . .	Male.	12 years.	Shot.	Diphtheria toxin. Maximum 500 cc. Bled 52 times. Last five bleedings at intervals of one week.	No amyloid degeneration. No hemorrhage.
LXXXII. .	Female.	14 years.	Shot.	Short treatment with diphtheria toxin. Rested. Bled 11 times. Frequency increased at last.	No amyloid degeneration. No hemorrhage.
XCIII. . .	Female.	10 years.	Shot.	No toxin. Bled 10 times at intervals of about one week.	No amyloid degeneration. No hemorrhage.

It is my purpose to consider these facts from several points of view which are more or less distinct from each other.

- I. The experimental production of amyloid.
- II. The effect of bleeding on normal horses and on horses with amyloid degeneration of the liver.
- III. The pathological anatomy of horses treated with diphtheria toxin and subjected to repeated bleedings.

I. *The experimental production of amyloid.*

Under this heading we have nine horses, ranging from nine to fifteen years in age, that have been injected with diphtheria toxin over considerable periods of time and bled at intervals. The horses after purchase are immunized by increasing doses of toxin, as rapidly as is possible without producing pronounced failure in health, until the antitoxic value of the serum reaches at least two hundred units. At the end of two months the horse is usually receiving about two hundred cubic centimeters of toxin at each injection. For the remaining period of the treatment the horse receives two or three toxin injections between consecutive bleedings which are about twenty-five days apart. The horse is not bled until ten days after an injection of toxin. After the antitoxin becomes strong enough for use the dosage of toxin is still further gradually increased. The maximum dosage over long periods of time is about five hundred cubic centimeters of a toxin whose minimum fatal dose is about .005 to .006 of a cubic centimeter. At each bleeding about five liters of blood per thousand pounds of weight are drawn. Under these conditions five-ninths of the animals autopsied have suffered with well marked amyloid degeneration of the liver or spleen or of both organs. The shortest period of treatment giving a positive result is two and a half years. Beyond this time the longer a horse is treated the more certain he is to suffer this degeneration. At three years and a half two of the horses were negative. Of four horses treated, four years and over, three showed amyloid

We have attempted to determine what part one factor in the treatment of these horses, that is the repeated bleeding, has in the development of the degeneration. The cases of horses 93 and 82 show that severe repeated bleedings at weekly intervals for ten weeks do not cause amyloid degeneration. The case of horse 56 shows that even when preceded by a three and one-half year period of mixed treatment five bleedings at intervals of one week do not develop the degeneration. So far as it goes the evidence is that the crude toxin injected is the factor of prime importance in the production of amyloid.

Microscopic examination shows that the distribution of the amyloid produced by this method is always the same with relation to the tissue and the individual cell. In all cases it appears in the liver, although in two it is more abundant in the spleen. These two are the only cases in which the spleen is affected. The horse which showed most amyloid in the spleen was treated for five years with typhoid toxin and for one year with diphtheria toxin. Whether this variation from the usual treatment accounts for the localization in the spleen rather than the liver seems doubtful in view of the case of horse No. 59 which under the influence of the diphtheria toxin developed more splenic than hepatic degeneration.

## II. *The effect of repeated bleeding.*

While the amyloid degeneration cannot be positively referred to any one factor in the treatment our results throw more light on the cause of the hemorrhage. As may be seen by reference to the table there is marked hemorrhage into the liver in three of the horses — two of them dying by rupture of the organ and intraperitoneal hemorrhage. All of these horses show amyloid degeneration of extreme grade in those portions of the liver which are hemorrhagic. In all there was amyloid throughout the liver and hemorrhage into parts. In the case of the two horses in which hemorrhage did not occur the amyloid degeneration is less severe. In one of them it is inconsiderable. In the horses showing amyloid throughout the organ and hemorrhage into portions

of it, the non-hemorrhagic areas show less amyloid than the hemorrhagic ones. None of the animals in our series had *hemorrhage without amyloid degeneration*. From these facts we conclude that amyloid degeneration in the liver of the horse is a serious predisposing factor toward hemorrhage into, and rupture of the organ. While we recognize the possibility that the same final result might be reached by another mechanism entirely, in our own cases the only precursor of the liver hemorrhages that can be demonstrated anatomically is amyloid degeneration.

Those who have noted the association of liver rupture with amyloid degeneration have ascribed it to the greater brittleness either of the liver tissue as a whole or of the affected vessel walls. The friability of the liver tissue was noticeable in our cases, but there seems to be another factor which may influence the hemorrhage. The hemorrhage, in fact, occurs only in those cases and in those parts of the liver where the amyloid degeneration is most extreme, and where the circulation through the sinusoids is seriously impeded or completely obstructed. This obstruction is always peripheral in the lobule and where it is practically complete the result is to throw the full force of any regurgitant wave from the right heart into the center of the lobule. The normal liver tissue of the horse is not very much stronger than that of smaller animals, and the heart is of course much more powerful. We have fulfilled in these cases the conditions for a hemorrhagic infarction of the liver as laid down by Chiari for human cases. In his cases the obstruction to the portal and peripheral lobular circulation was due to tumor metastasis. That this is the nature of the hemorrhagic lesions in one of our cases is shown by the histological examination of the liver of horse No. 3. Sections in several instances show diffuse infiltrating hemorrhages into the center of the liver lobules with extensive amyloid degeneration of the peripheral half. In one instance the wall of the central vein is ruptured, hyaline fragments of it remaining. In other instances the central vein is irregularly dilated to twice its normal size, the wall is eccentrically

thinned, but as yet unbroken. The central portions of the cords of cells are compressed. Amyloid degeneration of the peripheral portions of the lobule is extreme. Our other cases throw no light on the mechanism of the hemorrhage. The lesions are large. The tissue relations are greatly altered. The large masses of blood clot are bordered by liver tissue in many stages of necrosis, all of which may be ascribed to secondary tearing and pressure. Our cases do not show necrosis or thrombi in situations that would lead one to suspect that the hemorrhage was secondary to them.

We think that the hemorrhages in our cases resulted from the extension and fusion of multiple lobular hemorrhagic infarctions caused by amyloid degeneration so extreme as to obstruct the sinusoidal circulation at the periphery of the lobule. The process once started the extension is favored by the friability of the amyloid liver.

It is probable from our experience that disturbances of the circulation or perhaps undemonstrable changes in the composition of the blood incident to bleeding may determine the infarction and hemorrhage when the anatomical foundation is laid. The first horse lost here died early the next day after being bled. There had been a rise in temperature to one hundred and three degrees immediately after bleeding. There had also been a rise in temperature two weeks before at the time of the last toxin injection. A consideration of these facts led to the following experiments which have been carried out at intervals as the horses became available.

Horse XXX. — Treated five and one-half years. No toxin during the last six weeks. Drew three and one-half liters of blood four times at weekly intervals. One month after the fourth bleeding the temperature rose to one hundred degrees. The animal was sick. Four days later three liters of blood were drawn. Died in the night. Amyloid — hemorrhage — rupture of liver. Up to the time the frequent terminal bleedings were commenced the animal was in apparent good health, and there seems no reason to doubt that the frequent large bleedings were responsible for the acute development of the hemorrhagic lesions in the liver and that the last bleeding hastened the death of the horse in the same way.

Horse III. — Treated five years. Bled fifty-four times. During the period there was no marked rise in temperature. After the usual interval

was bled July 16. July 23 bled again. No symptoms. July 27, shot through the brain. Extreme amyloid degeneration of liver. Hemorrhage several inches in diameter beneath the capsule. Microscopic examination shows many hemorrhages into centers of lobules. Hemorrhages show no evidence of organization.

As a control on these horses that have shown extensive hemorrhages having an apparent relation to the bleeding there is one horse (No. 71) showing well marked amyloid disease but no hemorrhage. This animal was never bled oftener than once in twenty-five days.

Three cases, horses Nos. 22, 56 and 59, show that the long continued routine treatment with toxin and bleeding does not predispose to hemorrhage when the frequency of bleeding is increased, unless amyloid degeneration is produced. That is, the treated horse in which the liver remains normal anatomically behaves under frequent bleedings as does a normal animal.

In two experiments an attempt was made to determine the share of continued bleeding in the production of the whole disease.

Horse LXXXII. was treated and bled four times from August, 1905, to January, 1906. Toxin was then stopped. He was bled January 25, March 8, March 26, April 13, April 27, and then at intervals of five to nine days until the total number of bleedings was eleven. Shot. No lesion.

Horse XCIII. — Never had toxin. From April 27, 1906, to July 2, 1906, he was bled ten times, the intervals being about one week. Five and one-half liters of blood were drawn each time. July 3, shot. No lesion.

These experiments strengthen the view that the frequent bleedings only act to produce hemorrhage when preceded by amyloid degeneration. They of course throw but little light on what part is played by the long continued losses of blood by the horses.

Putting together these results we feel justified in drawing the conclusion that bleedings of the frequency recorded may acutely terminate the life of a horse if he has been under routine treatment in antitoxin production for a sufficient



length of time to give rise to extreme amyloid degeneration of a portion of the liver. Attention to this point might lead to a practical saving by prolonging the life of the valuable animals.

### III. *Pathological anatomy.*

Gross examination. — Important gross pathological lesions having any bearing on the present problem are limited to the spleen, liver, and peritoneal cavity.

The peritoneal cavity in the two horses that died was found to contain large quantities of dark blood-stained serum and uncoagulated blood. Large portions of the liver were found to consist chiefly of blood clot. Margining the clots were areas of liver tissue intermingled with smaller clots and portions of liver in which the structure is roughly preserved but with a diffuse infiltration of blood. Surrounding the clots and in portions not hemorrhagic there are areas in which the liver substance is very pale and waxy in appearance. The tissue is very friable. In one horse large masses consisting of a large connective tissue capsule and a central content of dark grumous fluid, were found. The liver is covered by the adherent omentum. On cut section the vessels are wide open and patulous.

Microscopic examination. — The material for histological examination came from the horses listed in the table. The horses unaffected have been considered as control material in a general way. In addition to these I have been able to examine the tissues of a number of horses recently autopsied in Montana under the supervision of Dr. Smith. These control studies have made it possible to limit the present description to the relatively gross changes — amyloid degeneration and hemorrhagic necrosis. All the more minute lesions and alterations are found in horses outside the antitoxin series as frequently as in it.

The autopsies have been performed as quickly as possible after death. One horse died in the night and the post-mortem was held eight hours later. In most cases the animals were killed and the section made at once.

Zenker's fluid has been the routine fixative. All the tissues have been examined fresh or after formaldehyde fixation for fat, staining with Sudan III. in the later case. The liver in two instances was fixed in absolute alcohol and stained for glycogen.

The routine stains used have been the eosin-methylene blue combination according to Mallory and Wright, and hematoxylin and eosin. Amyloid was recognized by the gross appearance and under the microscope by its optical characters, distribution, general staining reactions and, in some instances, by the specific stains on fixed tissues. The reactions to iodine, iodine followed by sulphuric acid, and to methyl violet have been readily obtained and have been quite permanent after Zenker's fixation. The reactions, however, are seldom obtained throughout the sections. Mallory's connective tissue stain and Von Gieson's picro-acid fuchsin followed by hematoxylin have been found most useful in determining the extent and exact distribution of the amyloid degeneration. All permanent section work was done after embedding in paraffine.

The tissues of the horse have been studied but little by modern histological methods. The frequency of lesions due to worms, the common presence of glycogenic infiltration of the liver, and the impossibility of fixing the blood in the vessels or studying it in unfixed material before it has undergone profound alterations are features so confusing that we think it worth while to consider them in detail.

There is an apparent increase in eosinophils in the tissue generally. This is especially prominent in the spleen and some lymph nodes. There are also local areas of connective tissue overgrowth in the lung and mesentery of some of the horses of our series which show enormous numbers of these cells. These are changes due to the invasion of the tissues with *Sclerostomum equinum*.

The livers which are normal on gross appearance and those least damaged by hemorrhage and amyloid degeneration, when fixed in Zenker's fluid and stained by any of the methods used, give an unusual picture. The cells are

swollen and have but little body. The cytoplasm is scant and coarsely granular. Definite vacuolation is not present, but in some instances a heavy cytoplasmic reticulum is seen. The cell wall is heavy and the nucleus stains lightly. The sinusoids are narrowed by the swollen cells. The changes in the parenchyma are probably those of a pronounced glyco-genic infiltration. Fatty d  generation has so rarely been encountered that it could not be responsible. Glycogen has been very abundant in the cases in which it has been tested for. The changes are more pronounced in our own horses, grain-fed and kept quiet, than in the range horses of the Montana series. There are changes in the blood within the vessels which are best considered along with similar changes in the spleen, adrenal, and kidneys.

In the spleen of all the horses there are focal areas of intense congestion and hemorrhage. These areas are usually in the pulp near the trabecul  . They are frequent in the trabecul  , and are nearly always found to some extent in the capsule. . In the cases in which the structure is nearest normal these areas are relatively few, perhaps one or two to a section and they show only congestion with dilatation of the sinusoids. To these may be superadded diffuse hemorrhage into the pulp. In one of our own horses the connective tissues in these hemorrhagic areas show hyaline necrosis and there is an infiltration of the area with polymorphonuclear leucocytes. The small acute hemorrhages without tissue reaction may perhaps be developed by a contraction of the spleen during the death agony. The trabecul   in the horse's spleen are very heavy and contain a great deal of involuntary muscle.

When the venules associated with these areas appear in sections they are usually found to contain a dense clump of red blood corpuscles. This clump of erythrocytes may extend into neighboring sinusoids. The clumped corpuscles stain with greater than normal intensity with eosin and phosphotungstic acid hematoxylin. They often seem to be held together by some extracellular substance, but the outline of the cells is usually distinct and can always be made

out. Typical hyaline thrombi have not been found in the spleen.

Similar dense clumps of corpuscles, and all transitions to them from the normal isolated corpuscles, are found occasionally in the kidney, and nearly always in the adrenal. In the latter organ they are usually unassociated with parenchymal or interstitial lesion. In the case of horse No. 93, a normal horse bled repeatedly, one adrenal shows such clumping in extreme degree in the periphery of the medullary portion. Here there are many hyaline thrombi, and a transition to the hyaline thrombus from the fused mass of red blood corpuscles is indicated. But whenever there are loose corpuscles or small clumps of corpuscles which can still be recognized as such there are also large numbers of coarse granules, possibly platelets. When the hyaline thrombus is well formed these are no longer to be seen. What becomes of them or why they disappear is uncertain. It is not impossible perhaps that they should flow together to form an imbedding substance for the red blood corpuscles. Associated with this thrombosis of the medullary veins is an intense congestion of the inner cortical zones. The opposite adrenal shows no acute lesion, but the remains of some hyaline thrombi almost absorbed are found in a few vessels. In the liver the sinusoids are frequently filled with irregular shadows varying from one-fourth to twice the size of the red blood corpuscle. They seem to be of erythrocytic origin. The better preserved erythrocytes have a tendency to clump and are sometimes fused. These changes are probably not an artifact due directly to the fixing fluid. They are present in tissue fixed in Zenker's fluid — formalin — acetone, methyl alcohol and absolute ethyl alcohol. The large and small shadows and the closely clumped hemoglobin containing red blood corpuscles have been seen several times in fresh specimens of these livers teased in normal salt solution. The changes in the blood in the liver sinusoids are more pronounced in the tissues of our own horses than in those of the western series, and this in spite of the fact that in certain instances in our own cases especial effort was made to get quick fixation of small blocks.

It is probable that the horse almost always has in the spleen, and frequently in other organs, small red blood corpuscle plugs in the capillaries and small veins, and that under favorable conditions these may progress to the formation of hyaline thrombi and give rise to lesions of microscopic or small macroscopic size. It is possible that this tendency to intravascular clumping has relation to the rapidity with which the horse corpuscles clump and settle in horse serum either diluted or undiluted outside the body. It seems impossible at the present time to decide which of these changes are due to post-mortem, and which to ante-mortem conditions except when the hyaline thrombus is fully developed or when there is some definite lesion of the surrounding tissues.

These microscopic thrombi and agglutination clumps and their relation to the focal lesions revealed by the microscope are of especial importance in the consideration of the pathological condition at present under discussion because of the frequency with which Pease and Pearce have observed thrombi in the animals of their series. In our own cases gross thrombi have not been found and it would modify our conception of the etiology of the condition if the microscopic examination warranted the assumption that there was an extensive thrombosis of the small vessels. This, however, is not the case.

The gross examination leads one to the conclusion that the important constant pathological changes in the immunized and bled horses are localized in the liver and spleen, particularly the liver. Careful microscopical examination of the tissue, from the animals, controlled by examination of a number of animals untreated or treated with wholly different agents, confirms this opinion.

The most striking of the essential lesions histologically, as well as macroscopically, are found in the liver. In the tissue of the five horses of our series which show abnormalities in the liver, it is possible to trace a series of changes beginning with slight amyloid degeneration which becomes extreme, a subsequent and probably consequent period of active

destruction, diffuse and massive destructive hemorrhages with cellular, lobular, and multilobular necroses, and finally, reparative processes affecting both interstitial tissues and parenchyma.

Of nine animals treated with toxin for a long time and bled repeatedly, which died or were killed, five show amyloid degeneration of the liver. One horse (71) shows only scattered small masses affecting single endothelial cells. In four the amyloid is in large amount. It affects in most important degree the outer one to two-thirds of the lobule. In the extreme cases small irregular extensions may reach to the region about the central vein. In every case the amyloid is placed in close relation to the endothelial cells lining the sinusoids. The line of division between cells can be traced through the mass of amyloid. There are thus formed units of the amyloid mass corresponding in size, and at first in position, to the endothelial cells affected. These units can be distinguished even where the degeneration is extreme and they become closely massed together. The nucleus of the endothelial cell and such parts of the membrane as remain visible, are always to the side toward the lumen of the vessel and are closely adherent to the amyloid. There is usually a well marked space between the outer surface of the amyloid masses and the adjacent liver cells. Projecting into this space there is frequently a radiating fringe of amyloid (see figure).

The space taken by the amyloid is gained at first by the atrophy of the liver cell. Later the sinusoid is encroached upon, but even when the amount of amyloid is extreme and the liver cell cords are reduced to a thin line the sinusoid is often patulous. Finally however, — and this is frequent in the cases showing hemorrhage, — the blood channels are wholly obstructed. The very narrow and obstructed sinusoids are only found at the periphery of the lobule.

The hemorrhagic areas of the liver usually show a large mass or masses of laminated clot intermingled with and bordered by fragments and lobules of liver tissue in various stages of necrosis and disorganization. Except in the late

cases when repair has begun there is never any well-defined cellular reaction in these border zones. Some of the border lobules are broken entirely away, others remain connected with the more normal liver tissue. Some show complete hyaline necroses. Others are diffusely infiltrated with blood and are partly necrotic. In one case well defined lines of hyaline central necrosis extend from the hemorrhagic to the more normal areas.

Usually there is no indication whatever of the path of the hemorrhage. But in the case of horse 3, in the regions somewhat removed from the gross hemorrhage, but where the amyloid degeneration is very marked, there frequently are found lobules whose sinusoids are occluded in the outer third. Many of these show diffuse hemorrhages into the central portions. Others have a much dilated, very thin walled central vein. The expansion of the vein may be sufficient to compress the central portion of the cords of cells back against those that are supported by the mass of amyloid. Occasionally the central vein is ruptured.

In one horse (No. 30) areas of old hemorrhage are found with the recent ones. In these cases the clot is wholly or partly organized, giving rise to areas of moderately dense cellular and fibrous connective tissue. In the spaces of the latter are found many endothelial cells containing blood pigment, and moderate numbers of wandering cells of other kinds — lymphocytes, plasma cells, and eosinophiles.

So far as my observation goes, the connective tissue growth always takes place from the periportal region. It is frequently possible to see areas of laminated clot touched on one side by apparently healthy liver tissue and, growing into it from another point, young connective tissue and blood vessels. On the side of the liver tissue there is no reaction whatever. The endothelial cells of the sinusoids or cells from the center of the lobule do not seem to take part in the process of repair.

Bile-duct regeneration or proliferation is found in this new connective tissue growth. In these cases, too, there is an evident attempt to regenerate liver parenchyma. Irregular

masses of cells having the character of liver cells, but without definite relation to sinusoids are found near the proliferating connective tissue. Occasional cells among these show definite karyokinetic figures. In a few places in the case of horse 3, where the amyloid degeneration is extreme but where there has been no hemorrhage, there is a proliferation of capillaries within the mass and these are followed by an increase of connective tissue. The process is not extensive enough to show the end result.

In one case (No. 3) there are in addition to amyloid degeneration very small focal hyaline hemorrhagic necroses. They seem to be gradually replaced by round cells and it is not likely that they influence the general disease, but they are interesting as being the only lesions found in these livers resembling in any way the lesions of acute diphtheria.

The spleen in two cases shows amyloid degeneration. In one of them the areas affected are small. They lie in the pulp just outside the Malpighian bodies. The degeneration, here as in the liver, seems to affect endothelial cells lining the sinusoids and these show little tendency to fusion. In the second case the degeneration is extreme, the whole pulp being transformed. The Malpighian bodies remain as rather narrow lines and small collections of cells. The sinusoids, not to be followed in section, must have been open, as they contain well preserved red blood cells.

The other organs show neither important nor constant lesions. A few of the glomeruli in the kidney of one horse show amyloid degeneration.

#### SUMMARY AND CONCLUSION.

Horses may be kept for a long time producing antitoxin if they are not bled more severely than five liters per one thousand pounds each three and one-half to four weeks. After the treatment has continued long enough to develop amyloid degeneration of the liver, increasing the frequency of the bleeding risks killing the animal. Some saving may result from the practical application of this knowledge to routine work.



Amyloid degeneration of the liver and less frequently of the spleen is produced in a majority of horses by the routine treatment with diphtheria toxin and repeated bleeding extending over a period of three years. The longer the treatment is continued the greater the certainty of the onset of the amyloid disease.

Horses with amyloid degeneration of the liver are very subject to hemorrhage into the liver substance and to rupture of the organ, with hemorrhage into the peritoneal cavity.

The hemorrhage extends more readily because of the great friability of the amyloid liver. It very possibly results from an extension of intralobular hemorrhagic infarctions which are probably directly induced in the already diseased liver by the disturbances in circulation incident to bleeding. When the hemorrhage does not break through the surface it is either encapsulated, resulting in a blood cyst, or absorbed and organized, resulting in an irregular increase in connective tissue in the liver. Regeneration of parenchymal and bile duct epithelium is attempted in the late cases.

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NOTE. — When this manuscript was prepared I overlooked the fact that the paper of Pease and Pearce referred to in the text (p. 451) had been published in *The Journal of Infectious Disease*, Vol. III., No. 4, June, 1906. They report the death of ten horses with rupture of the liver and hemo-peritoneum, and three horses killed at some stage of immunization. The horses had been treated with the toxic products of four species of bacterium and the results are with justice ascribed to the bacterial products, rather than the antiseptics and bouillon constituents which indeed could be definitely excluded as factors in several of their cases. The lesion underlying the liver rupture in all their cases was an acute general or local necrosis of the organ. This radical difference between their results and ours seems to depend on a difference in methods employed. Their treatment of the animals is evidently more vigorous than that employed in our laboratory. The doses of toxin are larger and the bleedings more severe. As a consequence, the animals do not live so long and the essentially chronic type of fundamental lesion seen in our cases does not have time to develop. They note two cases of amyloid degeneration, both in horses treated with diphtheria toxin. They are inclined to attribute this degeneration to the influence of the many abscesses which these horses suffered. While the abscesses may have hastened the process, our experience rules them out as an essential condition. Abscesses have been very rare with us. Venous thrombi — sometimes in the portal vein — were found in a number of their cases. They hesitate to draw the conclusion that the necroses were secondary to the vascular lesion. In this connection the frequency of thrombi in the blood vessels of the horse due to *sclerostomum equinum* should be noted. They note that in one of their cases death occurred immediately after bleeding. No information is given on this point in connection with the other cases. The case strengthens our idea that the disturbance of circulation incident to bleeding may induce the liver rupture when the anatomical foundation is laid. On the whole, the results in the two series of cases are complementary.

## DESCRIPTION OF PLATE XXIX.

AMYLOID DEGENERATION IN LIVER OF HORSE 3 (x 1,000 diameters).

The amyloid lies close to the sinusoids. There is a narrow space between the amyloid and liver cells with fine radiating prolongations of amyloid reaching into it. The sinusoids contain shadows of variable size and density.

[We thank Dr. S. T. Orton of the Boston City Hospital, who took the photograph for us.]





## THE APPLICATION OF PHYSICAL CHEMISTRY TO SERUM-PATHOLOGY.\*

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THE discovery that certain serums possess antitoxic and bactericidal properties, marked a new era in experimental therapeutics. Investigation was soon blocked, however, by failure to isolate the active principles of such serums in sufficient purity for chemical study. A knowledge of the chemical composition of these substances is essential to the ultimate progress of curative science.

Direct analysis being apparently impossible, recourse was had to indirect methods. The most promising of these indirect methods is the method of physical chemistry. It is the purpose of the present paper to examine certain attempts to apply physico-chemical laws in this field, and to point out fundamental conditions that must be fulfilled in future attempts.

### I. THE PARTITION COEFFICIENT.

What is probably the simplest physico-chemical law to be thus applied is the law governing the distribution of a soluble substance between two immiscible solvents. This law has been accurately determined for a number of simple organic and inorganic substances, and is expressible in mathematical terms.

To illustrate it, let us suppose that benzene and water are placed together in the same receptacle (Fig. 1), and that benzoic acid, which is soluble in both, is added to the mixture. How will the benzoic acid divide itself between the two solvents?

Benzoic acid dissolved in water has the chemical formula  $C_6H_5COOH$ . Dissolved in benzene, it polymerizes to form

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( $C_6H_5COOH$ )<sub>2</sub>. We should, therefore, expect, theoretically, that division would take place in accordance with the formula:

$$\frac{\text{Concentration* of benzoic acid in water}}{\sqrt[2]{\text{Concentration of benzoic acid in benzene}}} = K, \text{ a constant.}$$

This has been found experimentally to be true,  $K$  being approximately equal to 0.05.<sup>1</sup>

To make the formula concrete, let us suppose that 100 c.c. of benzene and 100 c.c. of water are placed together, and that 17.2

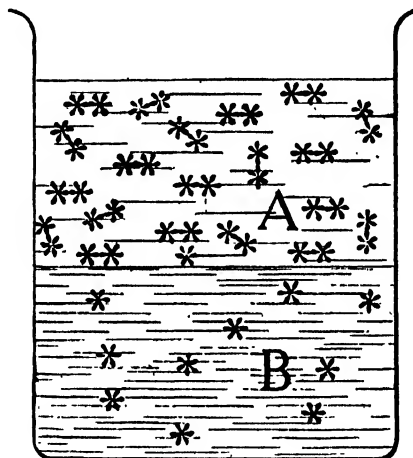


FIG. 1. THE PARTITION OF A DISSOLVED SUBSTANCE BETWEEN TWO IMMISCIBLE SOLVENTS.—A=Layer of benzene, containing double molecules of dissolved benzoic acid. B=Layer of water, containing single molecules of benzoic acid. The benzoic acid divided itself between the two solvents, so that

$$\frac{\text{Concentration of benzoic acid in water}}{\sqrt[2]{\text{Concentration of benzoic acid in benzene}}} = 0.03.$$

The constant, 0.03, is spoken of as the *partition coefficient*.

grams of benzoic acid are added to the mixture. How many grams of the benzoic acid will be held in solution by the water? How many by the benzene?

If  $x$  represents the number of grams of benzoic acid dissolved in the water, then  $(17.2 - x)$  will represent the number dissolved in the benzene. The concentration of the benzoic acid in water will equal:

$$\frac{x}{\text{volume of water}} = \frac{x}{100} \text{ grams per c.c.}$$

\* Number of grams per unit volume (1 c.c.).

The concentration of the benzoic acid in benzene will equal:

$$\frac{17.2-x}{\text{volume of benzene}} = \frac{17.2-x}{100} \text{ grams per c.c.}$$

Substituting these values in the above formula, we have:

$$\frac{\frac{x}{100}}{\sqrt[2]{\frac{17.2-x}{100}}} = 0.03,$$

from which

$$100x^2 + 9x = 154.8,$$

$$x = 1.2,$$

$$17.2 - x = 16.0.$$

Of the 17.2 grams added to the mixture, 1.2 grams will therefore be held in solution by the water and 16 grams by the benzene.

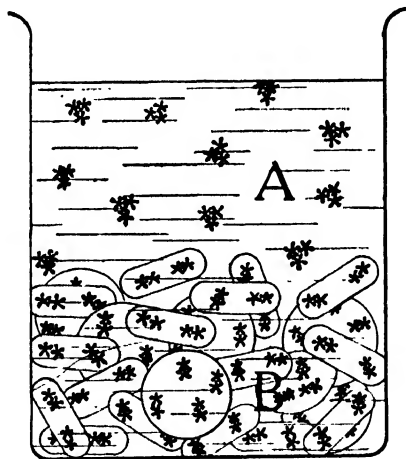


FIG. 2. THE PARTITION COEFFICIENT APPLIED TO SERUM PHENOMENA.—The specific thermostable substance of hemolytic serum is conceived to exist as double molecules when bound to corpuscles, but as triple molecules when free in the surrounding fluid. Division between fluid and corpuscles is thought to take place in accordance with the formula:

$$\frac{\sqrt[2]{\text{Concentration of bound thermostable substance}}}{\sqrt[3]{\text{Concentration of free thermostable substance}}} = K.$$

A—Supernatant fluid, containing the hypothetical triple molecules of the free thermostable substance.  
B—Settled corpuscles, containing the assumed double molecules of the bound thermostable substance.  
This conception can neither be proved nor disproved, due to the impossibility of applying direct analytical methods to absorption phenomena.

Applying this idea to serum phenomena, it is conceived, for example, that the specific thermostable substance of hemolytic

serum divides itself between corpuscles and surrounding fluid (Fig. 2) in accordance with a similar law. It is conceived that the thermostable substance exists as a double molecule when bound to corpuscles, but as a triple molecule when free in the surrounding fluid, and that division takes place in accordance with the formula:

$$\frac{\sqrt[3]{\text{Concentration of thermostable substance in corpuscles}}}{\sqrt[3]{\text{Concentration of thermostable substance in surrounding fluid}}} = K.^2$$

TABLE 1.

ATTEMPT TO APPLY THE PHYSICO-CHEMICAL PARTITION COEFFICIENT TO HEMOLYTIC SERUM.

Amount of free and bound thermostable substance determined experimentally, and  $K'$  calculated from the formula:

$$\frac{\sqrt[3]{\text{Bound thermostable substance}}}{\sqrt[3]{\text{Free thermostable substance}}} = K'.^*$$

The fact that  $K'$  is not a constant does not disprove the physico-chemical law, on account of a fundamental error in the experimental method. (Same data shown graphically in Figs. 3 and 4.)

Free Thermostable Substance	Bound Thermostable Substance	$K'$
4.22 C.C.	0.78 C.C.	37.5
3.28	0.72	28.8
2.66	0.84	11.0
1.66	0.84	4.7
1.16	0.84	2.3
0.79	0.71	1.7
0.64	0.61	1.9
0.42	0.58	0.9
0.39	0.41	2.2
0.29	0.31	2.8
0.27	0.13	33.2
0.17	0.03	1070.0

TABLE 2.

## THE SERUM PARADOX.

Data obtained experimentally, as in Table I. The data show, when certain amounts of serum are used, an apparent increase in thermostable substance, after contact with corpuscles. The table indicates a fundamental error in the analytical method. (See Fig. 5.)

Total Amount of Thermostable Substance Present in the Serum	Apparent-Amount Present after Contact with Corpuscles	Percentage
1.00 C.C.	0.55 C.C.	55
2.00	1.20	60
3.00	2.15	72
4.00	3.35	84
5.00	4.80	96
6.00	6.25	104
7.00	7.60	109
8.00	8.90	111
9.00	10.28	114
10.00	11.62	116

\*  $K'$  is a multiple of  $K$ , obtained by omitting to divide by volumes. Since the volumes are constant throughout the experiment, this omission introduces no error.



TABLE 3.

## NON-AGREEMENT OF DUPLICATE ANALYSES.

Analytical data from Fig. 6. Duplicate analyses show that after contact with corpuscles the serum contains apparently anywhere from 32 per cent to 77 per cent of the original amount of thermostable substance, the observed amount depending on the volume taken for analysis. This finding shows the impossibility of applying direct analytical methods to absorption phenomena.

Volume Analyzed	Apparent Thermostable Substance Found	Percentage
0.88 c.c.	0.270 c.c.	32
0.77	0.277	36
0.66	0.271	41
0.55	0.264	48
0.44	0.249	57
0.33	0.229	69
0.22	0.169	77

Can this be proved experimentally?

In attempting its proof,<sup>3</sup> data were obtained that do not agree with the formula (Table 1, Figs. 3 and 4), that is, data that do not

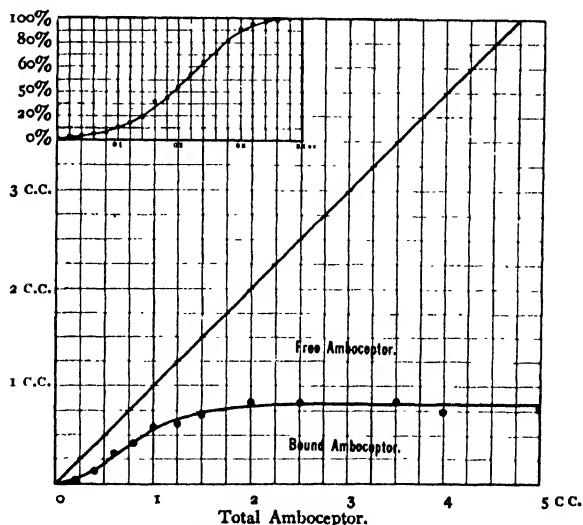


FIG. 3. ABSORPTION OF THE SPECIFIC THERMOSTABLE SUBSTANCE BY BLOOD CORPUSCLES.—Data from Table 1, represented graphically. The small curve in the upper part of the figure is the "Amboceptor Curve" used in the analysis.

give a constant value to  $K$ . The data, in certain cases, even lead to paradoxical conclusions, for the amount of thermostable substance present in the supernatant fluid, is at times apparently greater than

\* For the use of the "Amboceptor Curve" in analysis, see *Jour. Infect. Dis.*, 1905, 2, p. 471; *Centraltbl. f. Bakt.*, 40, p. 401.

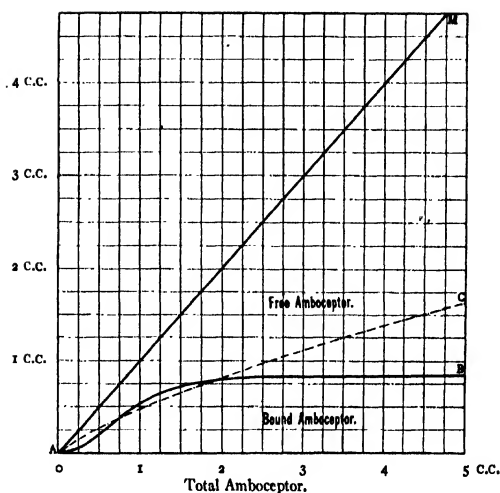


FIG. 4. OBSERVED ABSORPTION AND THEORETICAL ABSORPTION COMPARED.—Curve AB=observed absorption curve, as in Fig. 3. Curve AC=absorption curve calculated from the constant,  $K'=2$ . The fact that the two curves do not agree, does not disprove the physico-chemical law, on account of a fundamental error in the experimental method.

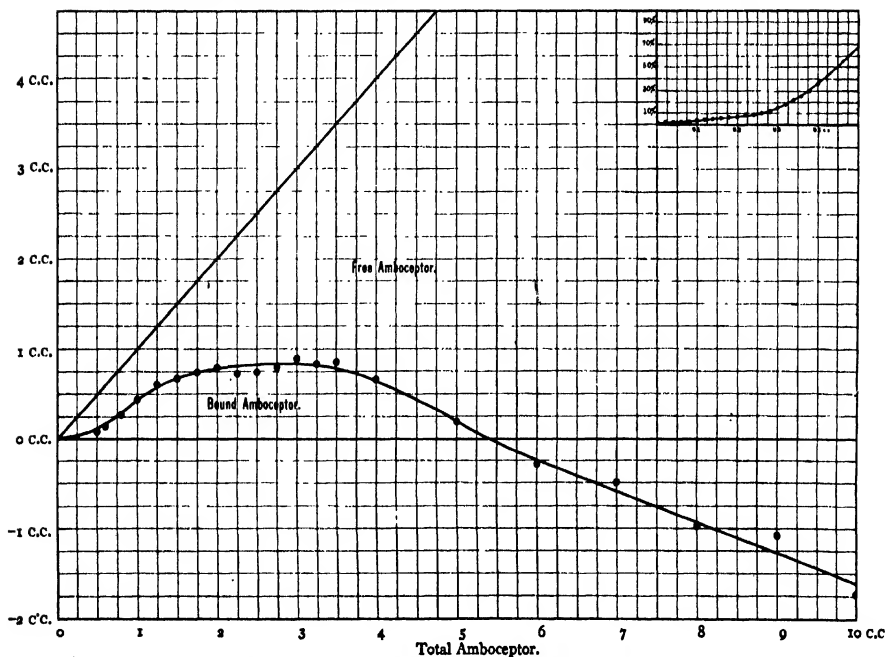


FIG. 5. THE SERUM PARADOX.—Data from Table 2 represented graphically. The data show the amount of free thermostable substance, at times apparently greater than the total amount of thermostable substance used in the experiment. The small curve in upper part of the figure is the "amboceptor curve" used in the analysis.

the total amount originally added to both fluid and corpuscles (Table 2, Fig. 5).

This paradoxical result is explained by the fact that heated hemolytic serum undergoes marked chemical changes when placed in contact with corpuscles<sup>4</sup> (Fig. 6).

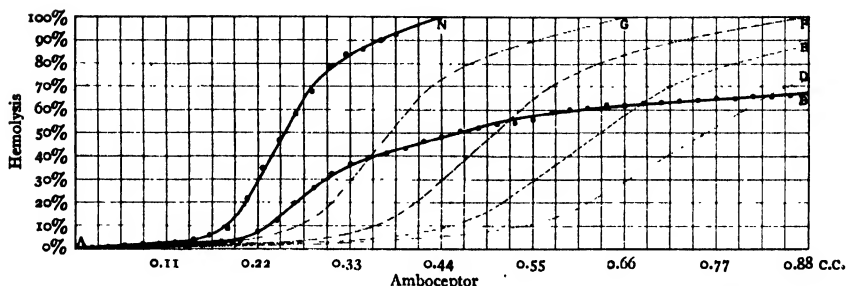


FIG. 6. QUALITATIVE CHANGE IN HEATED HEMOLYTIC SERUM AFTER CONTACT WITH CORPUSCLES. —Curve AN—curve showing the changes in hemolytic power as increasing amounts of thermostable substance (heated hemolytic serum), before contact with corpuscles, are added to a constant amount of thermostable substance (normal serum). Curve AB—curve obtained with the same thermostable substance after contact with corpuscles. Curve AB should be similar to one of the dotted curves of the figures, if the heated hemolytic serum suffered only a quantitative change during contact with corpuscles (AD=33½ per cent, AE=40 per cent, AF=50 per cent, AG=66½ per cent). The fact that AB crosses a number of these curves, shows that there is a change in the chemical composition of the serum as well. This change leads to non-agreement of duplicate analyses (see Table 3).

Occasionally, after contact when used in certain quantities the serum is hemolytically more active than originally. (Fig. 7).

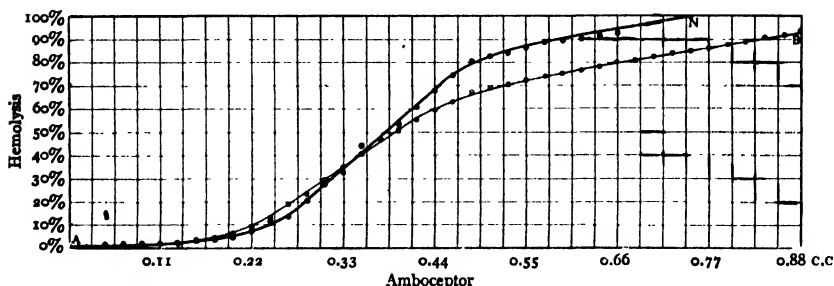


FIG. 7. QUALITATIVE CHANGES IN HEATED HEMOLYTIC SERUM, AFTER CONTACT WITH A MINIMAL NUMBER OF CORPUSCLES.—Curve AN—curve as in Fig. 6, showing the changes in hemolytic power as increasing amounts of the original thermostable substance (heated hemolytic serum) are added to a constant amount of thermostable substance. Curve AB—curve obtained with the same thermostable substance after contact with a minimal number of corpuscles. The fact that when used in certain amounts the thermostable substance after contact is apparently stronger than at first explains the paradoxical phenomenon previously noted. (See Table 2, Fig. 5.)

This chemical change is partly due to the fact that such serums contain, in addition to the specific thermostable substance, certain

non-specific auxilytic and antilytic substances<sup>5</sup> (Figs. 8, 9 and 10), the amount and nature of which are changed by contact with corpuscles.<sup>6</sup> Whether there is an independent change in the specific thermostable substance itself, or not, has not yet been determined.

Heated hemolytic serums, before and after contact with corpuscles, are not analytically comparable. It is consequently at present impossible to draw quantitative conclusions from serum experiments regarding the absorption of the specific thermostable substance. The proposed quantitative law cannot be proved. It cannot be disproved. Its proof or disproof will come only with future ability, either (1) to separate such serum into its individual components, or (2) to render all but the specific thermostable substance inactive.

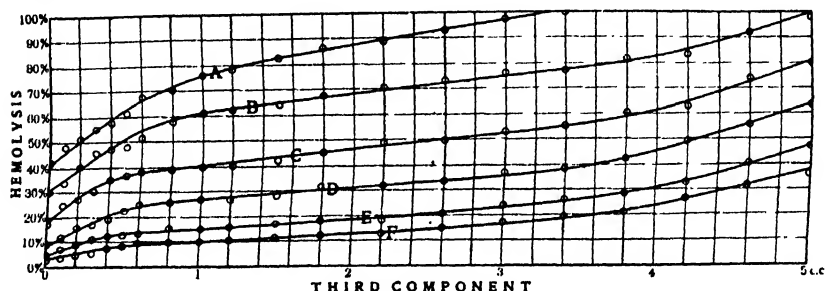


FIG. 8. THE AUXILYTIC ACTION OF CERTAIN NON-SPECIFIC SERUM COMPONENTS.—The curves show the changes in hemolytic power as increasing amounts of the non-specific substances are added to constant amounts of hemolytic serum. The constant hemolytic serum used for the different curves are, in themselves, capable of producing the following percentages of hemolysis: A=2 per cent, B=31 per cent, C=18 per cent, D=8 per cent, E=5 per cent, and F=3 per cent.

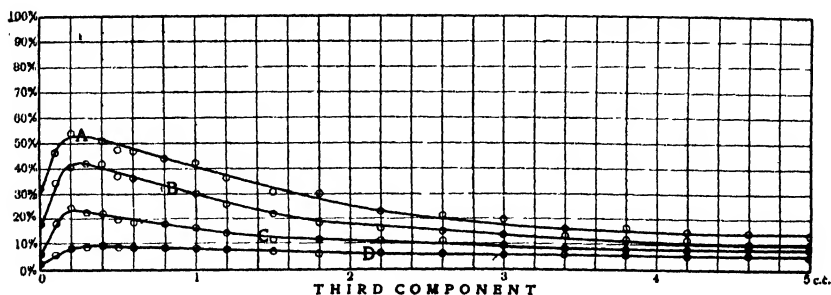


FIG. 9. THE ANTILYTIC ACTION OF CERTAIN NON-SPECIFIC SERUM COMPONENTS.—Curves, as in Fig. 8, showing changes in hemolytic power as increasing amounts of the non-specific substances are added to constant amounts of hemolytic serum. The constant hemolytic serum used for the different curves are, in themselves, capable of producing the following percentages of hemolysis: A=30 per cent, B=17 per cent, C=6 per cent, and D=2.5 per cent. The curves show an auxilytic action of the non-specific substances when used in small amounts, but their antilytic action when present in larger amounts.

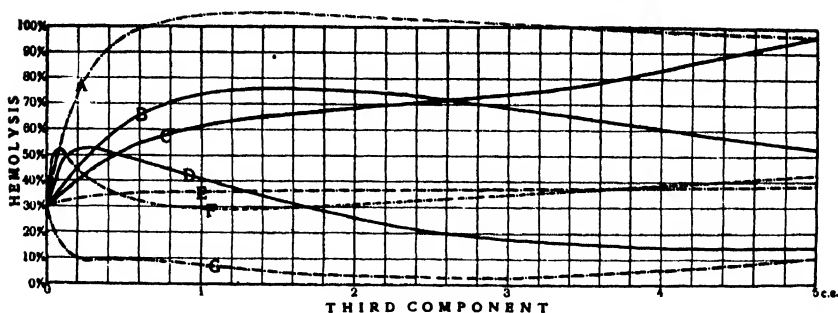


FIG. 10. THE ACTION OF THE NON-SPECIFIC COMPONENTS OF DIFFERENT SERUMS UNDER DIFFERENT EXPERIMENTAL CONDITIONS.—Sample curves, as in Figs. 8 and 9, superposed from different experiments, showing differences in the action of the non-specific substances in different normal serums, under different experimental conditions. All quantitative work heretofore done with hemolytic serum has neglected to take into account variations in these components. This fact alone renders all previous work inconclusive.

## II. REVERSIBLE REACTIONS.

A second physico-chemical law, whose application has been attempted to serum phenomena, is the law governing reversible reactions. This law also has been very accurately determined for a large number of simple organic and inorganic substances, and is likewise expressible in mathematical terms.

To illustrate it, let us suppose that common salt is dissolved in water (Fig. 11). As the molecules of the NaCl enter into solution they begin to decompose, forming free ions of Na and Cl. This ionization goes on at a definite rate, but is antagonized by a tendency of the atoms thus set free to recombine, forming anew molecular NaCl.

The ionization and recombination at first go on at different rates; but eventually a state of equilibrium is reached, in which the rates of dissociation and recombination exactly balance each other. When this is true, it follows theoretically that:

$$\frac{(\text{Concentration of Na ions}) \times (\text{Concentration of Cl ions})}{(\text{Concentration of the molecular NaCl})} = K.$$

This formula has been tested experimentally, and it has been found that for NaCl, at ordinary temperatures,  $K$  is approximately equal to 1, provided the concentration of the Na and Cl ions is expressed as the number of gram ions per liter, and the concentration of the molecular NaCl as the number of gram molecules per liter.

To make the law concrete, let us suppose that 117 grams of NaCl are dissolved in 1,000 c.c. of water. How much of the NaCl will exist in the molecular form? How much as free Na and Cl?

The molecular weight of NaCl is 58.5; 117 grams dissolved in 1,000 c.c. will therefore give a concentration of 2 gram-molecules

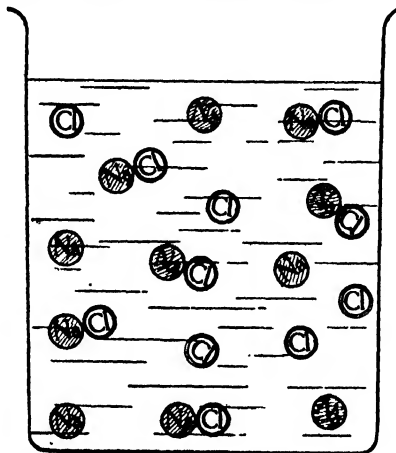
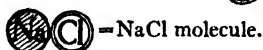
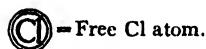
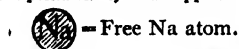


FIG. 11. THE IONIZATION OF SODIUM CHLORIDE.—A certain percentage of the NaCl molecules break up each minute, forming free Na and Cl atoms. A certain percentage of the Na and Cl atoms thus set free recombine, per minute, to form molecular NaCl. When equilibrium is established in the solution, the number of molecules decomposing each minute exactly equals the number recombining. The quantitative relation between the bound and free atoms is then expressed by the formula:

$$\frac{(\text{Concentration of free Na}) \times (\text{Concentration of free Cl})}{(\text{Concentration of molecular NaCl})} = K.$$

$K$  is found experimentally to be approximately equal to 1.



per liter. If, then,  $x$  represents the number of gram-molecules ionized,  $2 - x$  will represent the number remaining in the molecular form.

The ionization of  $x$  gram-molecules of NaCl will give rise to  $x$  gram-ions of Na and  $x$  gram-ions of Cl. Substituting these values in the above formula, we obtain:

$$\frac{x \times x}{2 - x} = 1,$$

from which

$$\begin{aligned} x^2 + x &= 2, \\ x &= 1. \end{aligned}$$

One gram-molecule of the Na Cl will therefore be ionized, leaving one gram-molecule as molecular NaCl. In other words, half, or 50 per cent, of the 117 grams will be dissociated.

If, in place of dissolving 117 grams, 44 grams of NaCl were used, about 67 per cent would be ionized. If but 14 grams, about 80 per cent; if only 6 grams, about 90 per cent; and so on.

a) *Hemolytic serum*.—This idea has been applied directly to serum phenomena. It has been conceived, for example, that the hemolytic substance of the hemolytic serum is a complex molecule formed by a union between the specific thermostable and the specific thermolabile substances of such serum. It has, further, been thought that this complex molecule, like the NaCl molecule above, is undergoing constant dissociation and recombination, and that equilibrium is established in accordance with the formula:

$$\frac{\left( \begin{array}{c} \text{Concentration of free} \\ \text{thermostable substance} \end{array} \right) \times \left( \begin{array}{c} \text{Concentration of free} \\ \text{thermolabile substance} \end{array} \right)}{\left( \begin{array}{c} \text{Concentration of the combined} \\ \text{thermostable-thermolabile molecule} \end{array} \right)} = K.7$$

Can this be proved experimentally?

In attempting its experimental verification, using a method proposed by its author,<sup>8</sup> data were obtained that do not agree with the formula.<sup>9</sup>

This lack of agreement does not necessarily disprove the formula, because the experimental method is at fault. It is experimentally impossible to vary the amount of either the specific thermostable substance or the specific thermolabile substance present in hemolytic serum, without producing simultaneous changes in the non-specific auxilytic and antilytic substances necessarily present.

This impossibility is due to the fact that the non-specific substances differ, under identical experimental conditions, both in amount and in chemical composition, in different serums<sup>10</sup> (Fig. 12).

On account of this difference, data that exactly agreed with the proposed formula would not prove the formula to be true. The experimental proof or disproof of the formula is at present impossible, and will come only with the discovery of a method either (1) to render these supplementary substances inactive, or (2) to make them identical in different serums.

b) *Diphtheria toxin*.—A second attempt to apply the law of reversible reactions to serum phenomena is the attempt to apply it to the interaction of diphtheria toxin and antitoxin. This interaction has been conceived to be similar to that between the Na atoms and Cl atoms above, the toxin and antitoxin molecules uniting to form a complex, dissociable, non-toxic compound. Equilibrium is thought to be established in accordance with the formula:

$$\frac{(\text{Concentration of free toxin}) \times (\text{Concentration of free antitoxin})}{(\text{Concentration of the combined toxin-antitoxin molecule})^2} = K.11$$

The data from which this conclusion was drawn were obtained by estimating the toxic effects on guinea-pigs of numerous toxin-

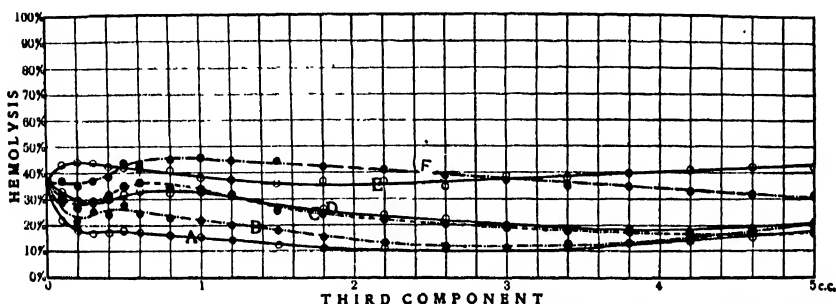


FIG. 12. COMPARISON OF NON-SPECIFIC COMPONENTS OF DIFFERENT SERUMS UNDER IDENTICAL EXPERIMENTAL CONDITIONS.—Curves, as in Figs. 8 to 10, showing changes in hemolytic power as increasing amounts of the non-specific substances of six different normal serums are added to the same constant amount of hemolytic serum. The constant hemolytic serum used in this experiment is in itself capable of producing 36 per cent hemolysis. While 0.2 c.c. of the non-specific substances of one serum (A) is capable of reducing the hemolysis to 17 per cent, the same amount of the non-specific substance of a second serum (E) will increase it to 45 per cent, and that of a third serum (F) will leave it unchanged. The curves show the impossibility of varying the amount of the thermostable or of the thermolabile substance in hemolytic serum without producing marked changes in hemolytic power, due solely to alterations in the non-specific serum components. This fact alone demonstrates the impossibility of testing experimentally the physico-chemical law proposed for the interaction of the specific thermostable and thermolabile substances.

antitoxin mixtures. Doses of the different mixtures having the same toxic action were assumed to contain the same amount of free toxin. Is this necessarily true?

If to a solution of NaCl there are added ions having an affinity for free Cl, not only will the new ions unite with the free Cl already present, but they will draw a certain amount of the bound Cl from the molecule NaCl as well. The number of Cl atoms thus split off will depend on the relative amounts of the Na atoms and the new ions, and on their relative affinities for free Cl.



For example, if Ag ions are added, they will enter into combination with the free Cl present, forming insoluble AgCl. New Cl ions will then split off from the molecular NaCl to restore equilibrium. The new Cl ions thus set free will in turn unite with the free Ag ions present, and this union will continue till either the Ag ions or the available Cl atoms are exhausted.

If, in place of Ag ions, ions are added that are capable of forming a dissociable compound with Cl, a similar robbing of the molecular NaCl will take place, but to a less extent. Thus, if to the 1,000 c.c. of NaCl solution above, 4 gram ions of *K* are added, the *K* will not only enter into relation with all of the free Cl present, but will draw 45 per cent of the bound Cl from the molecular NaCl as well; 8 gram-ions of *K* would draw over 60 per cent of the bound Cl, 12 gram-ions over 70 per cent, and so on.

Returning now to the guinea-pig experiment: we inject a toxin-antitoxin mixture into that animal. Toxins are known to have affinities for body cells.<sup>12</sup> If the hypothetical, dissociable, toxin-antitoxin compound really exists, the amount of toxin that would enter into chemical relation with the body cells would be, not only all of the free toxin already present in the solution, but a certain amount of the bound toxin as well, that would be split off from the toxin-antitoxin molecule in accordance with the laws of chemical equilibrium.

This additional amount of toxin might be large, or it might be small, depending upon the relative amounts of antitoxin and body cells and on their relative affinities for the toxin molecule. It could be negligible only under most exceptional circumstances,<sup>13</sup> the existence of which, to say the least, has not been proved.

The assumption that the toxic action of a toxin-antitoxin mixture depends entirely on the amount of free toxin present,<sup>14</sup> is unthinkable, if the proposed physico-chemical laws themselves hold good. Being fundamentally untrue, quantitative deductions from data obtained by its use are erroneous, regardless of the accuracy with which the data fit into the proposed formulae.

#### SUMMARY.

We can sum up the main conclusions of this paper as follows:

1. That the physico-chemical law proposed for the absorption of the specific thermostable substance of hemolytic serum by blood

corpuscles can neither be proved nor disproved, due to changes in the chemical nature of heated hemolytic serum after exposure to corpuscles, and the consequent impossibility of at present applying analytical methods to the phenomenon.

2. That the physico-chemical law proposed for the interaction of the specific thermostable and thermolabile substances of hemolytic serum can neither be proved nor disproved, due to the impossibility of varying the amount of either of these substances in a serum experiment, without producing indeterminate changes in the non-specific auxilytic and antilytic substances necessarily present in such serum.

3. That the physico-chemical law proposed for the interaction of diphtheria toxin and antitoxin can neither be proved nor disproved, due to the impossibility of measuring, by animal experiments, the amount of free toxin present in a toxin-antitoxin mixture, if the physico-chemical law itself holds good.

In conclusion it must be emphasized that the above facts do not prove that physical chemistry is not applicable to serum phenomena. They simply show that the phenomena here mentioned are too complex for accurate quantitative measurements by present experimental methods.

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- <sup>6</sup> Unpublished work now in progress.
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- <sup>8</sup> ARRHENIUS AND MADSEN, *Zeit. f. phys. Chemie*, 1903, 44, p. 45.
- <sup>9</sup> Unpublished work now in progress.
- <sup>10</sup> MANWARING, *Jour. Infect. Dis.*, 1906, 3, p. 654.
- <sup>11</sup> ARRHENIUS AND MADSEN, *Zeit. f. phys. Chemie*, 1903, 44, p. 7; MADSEN, *Brit. Med. Jour.*, 1904, 2, p. 568.
- <sup>12</sup> WASSERMAN, *Zeit. f. Hyg.*, 1901, 36, p. 172.
- <sup>13</sup> MANWARING, *Jour. Infect. Dis.*, 1906, 3, p. 645.
- <sup>14</sup> ARRHENIUS AND MADSEN, *Zeit. f. phys. Chemie*, 1903, 44, p. 44.

# THE CULTIVATION OF *SPIRILLUM* *OBERMEIERI*.\*

## PRELIMINARY NOTE.

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We have heretofore applied the term *Spirillum obermeieri* to the organism isolated by Dr. Charles Norris, from a case of relapsing fever which occurred in New York, in the belief that it was identical with that described by Obermeier in 1873. We will continue to use this term for the present, although, as will be shown, there is reason to believe that the eastern relapsing fever is not identical with the American disease. When further evidence is obtained regarding the non-identity of the organisms found in these regions it may be necessary to restrict the use of the term *Spirillum obermeieri* to the organism found in the eastern disease.

On the other hand, the African relapsing fever, known also as tick fever, has been shown conclusively to be due to a different organism, the *Spirillum duttoni*. As far as we know, the first observation on this organism was made by Dr. D. Nabarro, of the Sleeping Sickness Commission, who noted the presence of spirilla in the blood of a patient in Uganda as early as August, 1903, but this fact was not published until 1905. In November, 1904, the presence of spirilla in tick fever was announced by Ross and Milne and also by Dutton and Todd. This organism was brought to England by Todd, and to Germany by Koch, and has since been studied by several workers.

In our preliminary note<sup>1</sup> we pointed out the probability that tick and relapsing fevers were two distinct dis-

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\* From the Hygienic Laboratory, University of Michigan. This investigation has been aided by a grant from the Rockefeller Institute for Medical Research.

1. THE JOURNAL, Jan. 18, 1906.

eases, due to different species of spirilla, and in the main paper<sup>2</sup> it was definitely shown that these two organisms were distinct species, and at that time we named the spirochete of tick fever *Spirillum duttoni*. Our conclusion regarding the specific difference of the two organisms was based on a comparison of (1) the animal reactions of the New York spirillum, as ascertained by Norris and his co-workers and by ourselves, with those described by Dutton and Todd and later by Breinl and Kinghorn; (2) the morphologic characteristics presented by our organism with those of the tick fever spirillum in specimens sent by Dr. Todd, and (3) the arrangement of flagella as demonstrated in this laboratory for the New York spirillum and by Zettnow for that of tick fever. Our belief that a full confirmation of this view would be afforded when cross experiments were made with sera of animals immunized to these two spirilla has been realized by the subsequent work of Breinl<sup>3</sup> and by still more recent tests made with our serum by Dr. Schilling at the Institute for Infectious Diseases in Berlin.

It will be seen from the above facts that a recent note made by Breinl and Kinghorn<sup>4</sup> and implying that our conclusion was reached merely from "a study of the two slides sent from these laboratories and of the few experiments given by Dutton and Todd" is not a fair statement of fact. In his paper of June 16, Breinl suggested the specific name "*duttoni*," the same as used previously by ourselves. We have employed the generic name *Spirillum* in preference to *Spirocheta* for the reason that the type species of the latter genus, *Sp. plicatilis*, was said by Schaudinn to possess an undulating membrane, but no flagella. Furthermore, the term *Spirillum* expresses our view regarding the bacterial nature of these organisms.

In our article of last May, a brief discussion is given to the relation of the New York spirochete to that of the relapsing fever met with in Bombay, and the statement is made that the evidence on hand points to the existence

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2. Studies on *Spirillum Obermeyer* and Related Organisms, Jour. of Infect. Dis., May, 1906, vol. iii, pp. 291-393.

3. The Specific Nature of the Spirocheta of the African Tick Fever, The Lancet, June, 16, 1906.

4. A. Breinl and A. Kinghorn: An Experimental Study of the Parasite of the African Tick Fever (*Spirocheta duttoni*), Memoir XXI, The Liverpool School of Tropical Medicine, September, 1906.

of three relapsing fevers in man. It is unnecessary to recapitulate the reasons which led us to take this view, but it will be of interest to present in this connection some observations which have been made by Captain I. P. Mackie, I.M.S., and communicated by letter to one of us (Novy). It is generally understood, as a result of the studies of the earlier workers, that the spirillum of relapsing fever does not infect animals other than the monkey. Captain Mackie has produced the disease in rabbits, white English rats, black Bombay rats and in mice, but failed to infect pigeons and goats. In these animals the spirilla appear in less than 24 hours and sometimes disappear in less than 48 hours, but often increase to 56 or more hours. They never become numerous and do not reappear; that is, there is no relapse. These results are in accord with those of Gabritschewsky. The very scanty number of spirilla in his rats is in striking contrast with the result obtained with our *Sp. obermeieri* and with *Sp. duttoni*. Moreover, tests made by Captain Mackie with some hyperimmune serum which we sent him showed that it possessed no agglutinating, germicidal or lysogenic action even in dilutions of 1 to 10. Control tests with some of this serum were made in Ann Arbor, at about the same time as the Bombay tests, and these showed that the serum possessed undiminished activity toward our spirillum. The latter was promptly agglutinated by the serum in dilutions of 1 to 10 and even 1 to 200. Unless it is assumed that our serum had lost its properties while in transit, especially in going through the Red Sea, it would appear that the Bombay relapsing fever is etiologically different from that of New York. The question of the identity of these organisms will be investigated further and we hope to be able to make direct comparisons of the living organisms.

#### CULTIVATION EXPERIMENTS.

The *Spirillum obermeieri* has been maintained in this laboratory since November, 1905, by consecutive passage through rats. Although during this time many hundreds of attempts to secure cultures on artificial media have been made, they have given uniformly negative results. In the defibrinated blood of infected rats the spirilla retain their vitality for a variable length of time, depending on the stage of the disease during which the blood is drawn. If drawn during the decline stage,

that is to say, at a time when the organisms have reached their maximum and are beginning to decrease in numbers, the spirilla will often die out in less than 24 hours. This is due, as we have shown, to the presence of specific germicidal bodies. On the other hand, in "onset blood" drawn during the early stage of the disease, the spirilla may live for several weeks. Thus we have seen living spirilla in such blood kept for 30 to 37 days and have been able to infect rats with blood kept for 40 days. Moreover, we have been able to make use of this fact in shipping the virus to distant points, to Dr. Todd at Liverpool and to Professor C. Fraenkel at Halle.

In our first series of attempts at cultivating the spirilla on blood agar we were, as a rule, unable to keep the organisms alive for more than two or three days. Since then, however, we have been somewhat more successful and have kept them on blood agar for 22 to 28 days, and in some experiments now in progress they are still alive and numerous on the thirtieth day. As yet, however, no evidence has been obtained of actual multiplication *in vitro*. The organisms which are found to persist we prefer to regard as mere survivals until actual subcultures have been obtained.

The successful results obtained by Levaditi in the cultivation of *Sp. gallinarum*, *Sp. duttoni* and *Sp. refringens* in collodium sacs led us to apply this method to our spirillum. With this object in view, the collodium sacs were filled with rat or rabbit blood, or corresponding sera, heated and unheated, and after inoculation with spirilla blood these sacs were placed in the peritoneal cavity of rabbits. After three to seven days the sacs were removed and contents were examined with negative results. Apparently the rabbit is unsuited for sac cultures.

We were finally led to make the trials under conditions approaching the natural state as much as possible. For this purpose the collodium sacs were filled with uncoagulated rat blood and after inoculation were placed at once in the peritoneal cavity of a white rat. Three days later, on removal, the sacs were found to contain active spirilla and in increased numbers. From the sacs, transplants were made to new ones and the result was equally satisfactory. The spirilla were found to be in an extremely active condition and were undoubtedly multiplying.

From this time on the transplantations were made regularly, every three or four days, from sac to sac. After a few passages the uncoagulated blood was replaced by defibrinated rat blood or by rat serum. Defibrinated rabbit blood has also been employed to some extent, but whether it will continue to be a favorable medium we are unable to state. Two sacs were inoculated each time and placed in the peritoneal cavity of a rat. Each sac had a capacity of from 2.5 to 3.0 c.c. and was sealed so as to leave within as little air as possible. It is a noteworthy fact that on removal from the rat the sacs are invariably greatly distended as a result of osmotic changes. Furthermore, the air which was originally present is in large part and at times wholly absorbed.

Since October 13 the spirilla have been carried through twenty consecutive passages in sixty-eight days, and presumably they can be kept multiplying under these conditions indefinitely. The spirilla in the sac culture are never as numerous as in the blood of rats. They rarely exceed more than 5 to 10 per field of the  $1/12$ th inch objective, as contrasted with several hundred per field met with in the blood of rats during the maximum period of infection. The inoculation of the sac contents (blood or serum) into rats, it is interesting to note, is followed by a mild infection in which the spirilla are not much more numerous than in the sacs. Moreover, in such infection they persist for a day or two longer than is the case with the active virus.

When the sac is allowed to remain in the rat for seven days the spirilla decrease greatly in numbers and may even disappear. In the opened culture sacs after removal from the rat, and kept at room temperature, the spirilla die out in a day or two.

Throughout this series the spirilla have preserved their form unchanged. They appear either as single cells (8 microns) or of double length (16 microns), but at times even longer spirals are found. The latter are the result of end-to-end union by means of flagella, as we have heretofore shown. As in the case of blood preparations, no evidence is observed of division other than transverse. One observation in this connection is deserving of special emphasis owing to its bearing on the question as to whether spirochetes multiply by transverse or longitudinal division. In these cultures it is

not unusual to find short spirals of two or three turns, and from 4 to 6 microns in length. These may occur singly or in pairs (8 to 12 microns long), showing the pale division zone. The width of the short form is the same as that of the longer cells. The occurrence of these short spirals is readily explainable as the result of transverse division. It may further be stated that the cultural spirals usually stain solid by the Romanowsky method, but at times they may show granulations which to some extent may be due to granules deposited from the medium.

#### SAC CULTURES IN RAT SERUM.

In view of the fact that Prowazek and others are inclined to consider spirochetes as protozoa and as cell parasites, it was desirable to ascertain whether or not the spirilla could be maintained in active multiplication in a clear serum. Accordingly, the spirilla were inoculated into rat serum, completely freed from corpuscles by centrifugation. Up to the present time we have effected seven consecutive passages in such serum in the space of twenty-four days. At each passage a control sac containing defibrinated rat blood was placed in the rat. The serum cultures, although totally devoid of corpuscles, were in every respect as rich in spirilla as the blood cultures. The conclusion to be deduced from these experiments is that multiplication of spirilla may take place without any intracellular stage. The occasional presence of spirilla in a cell is to be regarded as an accident rather than as an expression of an unrecognized cycle.

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OBSERVATIONS ON THE CYTOLOGY OF MULTIPLE  
NON-INFLAMMATORY NECROSIS OF THE LIVER  
AND ON CERTAIN RELATED DEGENERATIVE  
CHANGES IN CELLS.

By DOUGLAS SYMMERS.

# OBSERVATIONS ON THE CYTOLOGY OF MULTIPLE NON-INFLAMMATORY NECROSIS OF THE LIVER AND ON CERTAIN RELATED DEGENERATIVE CHANGES IN CELLS.<sup>1</sup>

By DOUGLAS SYMMERS.

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There has been described recently from this laboratory<sup>2</sup> an independent affection of the liver characterized anatomically by "multiple, irregular, circumscribed solution of liver cells without parenchymatous degeneration or coagulation necrosis, and associated with a corresponding lobular blood and bile stasis in the affected areas. It appeared that the protoplasm and later the nucleus of the cells simply wasted and dissolved, leaving a more or less well preserved reticulum with stagnant blood and bile." The condition has been observed here six times (two unpublished cases) and seems to be characterized clinically by jaundice and mental symptoms, usually delirium, followed by a rapidly fatal issue. To the lesion as a whole Oertel has applied the term "multiple non-inflammatory necrosis of the liver with jaundice." The cellular changes he designates "cytolysis."

While several observers, notably Flexner in this country and Fr. Muller in Germany, correlating chemical with morphological findings, have sought to explain certain pathological processes on the basis of the property of certain cells to elaborate digestive ferments, practically no work has been done on the structural changes brought about in the tissues by the action of such substances. Hauser<sup>3</sup> in 1886 kept small pieces of liver under aseptic conditions for varying lengths of time. The specimens gradually softened.

<sup>1</sup> This study was aided by an appropriation from the Rockefeller Institute for Medical Research.

<sup>2</sup> *Jour. Med. Research*, 1904, xii, 75. *Jour. Exper. Med.*, 1906, viii, 103. *Collected Proc. N. Y. Path. Soc.*, new series, 1905-6, v, 143.

<sup>3</sup> *Arch. für exper. Path. u. Pharm.*, 1886, xx, 162.

Microscopically the cell form was found to be preserved to a late date, the nuclei were degenerate and the cytoplasm appeared granular. Waldvogel<sup>4</sup> recently repeated these experiments with like results. Both these observers, however, were working on problems which did not intimately concern the morphology of autolysis and consequently their description of the cellular changes is lacking in detail.

The present communication has to do, first, with a detailed description of the cell changes in multiple non-inflammatory necrosis of the liver and with related cell changes in other conditions, and, second, with the description of a variety of cell degeneration likewise affecting the liver and, I believe, generically allied to that of Oertel's lesion but presenting distinctly different characteristics, both general and cytological.

In multiple non-inflammatory necrosis the individual liver cells are large and, although irregular in outline, the various forms are well preserved. The nuclei are relatively large and occupy, as a rule, a central position in the cell body, being pushed aside only in those instances in which the cytoplasm has been the seat of fat deposit. The cytoplasm appears pale and washed out and stains poorly. The nucleus, relatively much richer in substance, stains correspondingly deeply; its shape for the most part is well preserved and the nuclear membrane appears to be intact and stains deeply, while immediately within the membrane are numerous large and small, deeply staining granules which follow the circumference of the nucleus. The center of the nucleus is pale and turbid and not infrequently is traversed by fine chromatin bands which appear to radiate from the relatively large, rounded, deeply staining nucleolus or nucleoli. In those cells in which the nucleus appears only slightly disintegrated the cytoplasm is correspondingly well preserved.

The change in the cytoplasm appears primarily to be a simple solution of the hyaloplasm with retention of the spongioplastic network, giving the cell a finely honeycombed appearance. In places the meshes of the spongioplasm are partly occupied by minute bile particles; in other places certain alveoli seem to have coalesced to

<sup>4</sup> *Virchow's Arch.*, 1904, clxxvii, 1; *Deut. Arch. für klin. Med.*, 1905, lxxxii, 437.

form a large vacuole for the reception of aggregations of such particles. In other places the cytoplasm shows large vacuoles which, in fresh sections stained in Sudan III, reveal the presence of neutral fats.

A further step in the destruction of the cell after the hyaloplasm has been dissolved out is represented by shrinkage of the spongio-plasm, followed by enlargement and distortion of the cell body and partial or complete nuclear destruction. The fine spongioplastic network may be retained in places and lost in others. In the end all that remains is a wrinkled but otherwise intact cell membrane enclosing, in some instances, a few coarse, irregularly distributed reticular fragments and occasional fat droplets or aggregations of bile.

It is found, however, that cell changes simulating those just described are not confined to multiple non-inflammatory necrosis of the liver. In the exudate in resolving pneumonia, in the cells that lie free in the alveoli in chronic cyanosis of the lungs, and in cerebral softening, similar structural changes are apparent, brought about, it is believed, by solvent action.

The solution of the red cells can be traced both in the capillaries and smaller vessels and in the alveoli, especially in chronic cyanosis of the lungs. The first change appears to consist of partial solution of the hæmoglobin manifested by a diminution of the avidity with which the cell takes up the acid stain. The corpuscle stains a dull pink or dirty yellow and becomes opaquely granular. From this point the staining qualities can be traced through certain gradations in color until finally the cell stains very faintly or not at all, appearing as a definitely rounded body with an intact cell membrane which encloses a pinkish or bluish or grayish opaquely granular material, the cell varying in diameter from five to twelve microns or even more. Finally the altered cell substance is completely dissolved out and all that remains is the cell membrane. This structure frequently retains its rounded shape, but occasionally appears wrinkled—the “shadow” or “ghost” cell. In the pulmonary alveolus the pale, definitely rounded, non-nucleated cells constitute a characteristic feature, the peculiar constitution of the cytoplasm being very striking. It is apparent, also, that after being extruded into the

alveolus the corpuscle often increases in size, an effect due, no doubt, to a variety of factors.

In the white cells certain individual morphological variations are presented. The leucocytes earliest affected are comparable to the large lymphocytes; the polymorphonuclear cells, while not infrequently involved, appear to be relatively immune or at least more resistant. The first visible change in the leucocytes consists of swelling of the cytoplasm, which becomes cloudy and stains a dull red with eosin. It then appears to lose entirely or almost entirely its affinity for the acid dye, staining faintly blue or pink. The cells become more or less irregular both in shape and size, although the continuity of the enclosing membrane is practically always preserved. At this stage the cytoplasm shows a distinct reticular arrangement with the formation of empty alveoli. The nuclei display various degrees of disintegration and are much distorted. The further destruction of the cell may be accomplished, however, in different ways and the various steps cannot well be followed. Sometimes the cytoplasm at the periphery is lost, a few irregular clumps being arranged around the rapidly disintegrating nucleus. Or the nucleus may be found lying next the cell membrane, where frequently it becomes flattened or crescentic in outline, while the cell body is made up of irregularly scattered reticular remnants. Finally the nucleus is lost and the cell appears either as an irregularly rounded body enclosing a few reticular fragments or as a mere shell.

In chronic encephalitis with extensive softening the leucocytes in the softened areas and, to a less extent, those in the better preserved parts of the brain, show all varieties of the degenerative changes just described that lead to the exposure of the cell reticulum. In addition there are to be seen numbers of large, very pale leucocytes whose outline is irregular but whose cell membrane is preserved. The cytoplasm appears to be made up of opaque, structureless material and the nucleus is markedly degenerate. In some of these cells the nucleus has disappeared and the cell membrane encloses opaque, amorphous material only, which may be scattered evenly over the surface or arranged irregularly in clumps. In still other cells the membrane encloses nuclear fragments and small

collections of debris. In the end the cell membrane alone remains.

This peculiar appearance of the cell may be likened to that of the cytolyzed red cells already described, or more aptly still to certain structural changes in the liver cells to be described later.

Related changes can be followed in the alveolar epithelium in pneumonia and in chronic cyanosis of the lungs. These cells, as they lie attached to or partially detached from the alveolar wall, appear as large, palely staining, flattened or elongated bodies with pale, rounded or oval nuclei which enclose rather deeply staining nucleoli. The cytoplasm presents a distinct reticulum and the hyaloplasm is not visible. After the cell is completely detached and lies free in the alveolus it often becomes swollen and irregular in shape, but retains otherwise the general characteristics just described. Its identification in this position can usually be determined by the fact that its nucleus encloses a nucleolus, by the shape of the cell and by the occurrence of large, golden brown pigment particles, although similar pigment masses now and then can be seen lying upon or within cytolyzing leucocytes.

In connection with the general question of cytolysis I wish to draw attention to a form of cell degeneration occurring in the liver, for which, assuming that the morphological interpretation is correct, because of the character of the cellular changes presented, I would suggest the term "cytolysis granulosa." Since the condition was first detected I have looked for it in about two hundred cases and have been able to find it present seven times as a widely spread, diffuse affection and several times as a more restricted process. Neither the clinical histories of the respective patients nor the microscopical findings in the different organs throw much light on the changes in the liver cells. Three patients presented well-marked anatomical evidences of syphilis. The gross appearance of the liver was not regarded in any case as at all remarkable.

In three instances the autopsy was performed within four or five hours after death. Specimens were preserved in 5 per cent. formalin solution and examined in frozen or celloidin and paraffin sections. The change appears to show to best advantage when sections are deeply stained in Böhmer's hæmotoxylin and examined under the oil immersion lens.

In this condition the liver cells appear for the most part to be arranged in definite rows, closely packed together; the cell outline is unusually distinct and the normal shape almost always is retained. The intercolumnar vascular and lymphatic channels are wide. Under the oil immersion lens the cells everywhere appear pale and of average size. The cell membrane stains deeply and stands out prominently against the pale cytoplasm. The nucleus almost uniformly is enlarged out of proportion to the size of the cell as a whole and is usually rounded, occasionally indented, in most cells lying in the center, in some being pushed to one side. In what seems to be the initial step in the process the cytoplasm looks pale and made up of turbid, granular looking, structureless material evenly but thinly distributed over the surface of the cell, and composed apparently of a combination of altered hyaloplasm and broken-down reticulum. The nucleus now shows beginning central turbidity with small chromatin granules arranged peripherally. A nucleolus—sometimes more than one—may be present. In cells still more badly damaged this hazy, dirty looking material is arranged in irregularly scattered clumps, while between the clumps are finer, hazy, granular looking particles. At this stage the nucleus is very pale and the center very turbid, but the nuclear membrane still stains deeply and is intact. A few small chromatin granules are visible within the body of the nucleus. Toward the end the nucleus shrinks in size and retreats toward the periphery of the cell where it seems to be supported partly by the cell membrane and partly by clumps of debris; its center now is extremely turbid but the enclosing membrane still is very definitely outlined. Finally, the cell contents disappear completely and the cell membrane alone remains.

In most cases moderate bile imbibition is to be observed. It occurs in two forms: First, as very finely divided particles scattered over certain cells and, second, as aggregations of such particles lying in large vacuoles in the cytoplasm. The vascular and lymphatic channels lying between the rows of liver cells are usually greatly dilated, their endothelial lining being easily distinguished. The vascular channels contain a few pale red cells, shadow corpuscles, small numbers of leucocytes and debris. Sections stained in Sudan III reveal, in most cases, fat in small amount, appearing

mostly as rather large rounded masses, but occasionally as very minute particles lying in the cytoplasm.

The peculiar material replacing the cytoplasm of the liver cells is insoluble in chloroform, ether, alcohol, equal parts of ether and alcohol, 1 per cent. sodium hydrate solution and 2 per cent. acetic acid solution. The last substance, however, renders it cloudy, so that the cell membrane is obscured. It is unaffected by prolonged exposure to 1 per cent. osmic acid solution, except to a very slight degree, a few small, blackish spots appearing here and there.

From these observations the following conclusions may be drawn :

1. Those pathological processes that appear to depend upon cytolysis present at least two definite types of structural changes in cells—the one characterized by rapid, simple solution of the hyaloplasm with preservation of the cell reticulum as, for example, in multiple non-inflammatory necrosis of the liver; the other by alteration of the hyaloplasm and reticulum with the formation of amorphous material, both the cell and nuclear membranes remaining very resistant, as in the liver cells just described. Extreme tenacity of the cell membrane appears to be the most constant single feature of all forms of cytolysis thus far recognized.

2. Polychromatophilia is associated with solution of the hæmoglobin of the red cells, especially in those conditions which are attended by destruction of blood. It appears not improbable that this peculiar reaction of the cell may represent a physiological as well as a pathological condition. As a physiological process it is conceivable that the polychromatophilia apparent in the earlier developmental stage of the red cells represents an attempt on the part of the organism to dispose of an excess of such cells by solution or that the cells have not yet received their full supply of hæmoglobin.

In closing I wish to express my appreciation of the interest shown in this work by Dr. Horst Oertel.



## ON THE QUANTITATIVE ESTIMATION OF TRYPTOPHAN IN PROTEIN CLEAVAGE PRODUCTS.

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The method of Hopkins and Cole<sup>1</sup> made possible the isolation of tryptophan from the other protein cleavage products. The methods of preparation of the pure substance always lead to appreciable loss and the yield of the pure substance does not furnish exact information regarding the real quantity of this constituent in the protein molecule. It seemed, therefore, desirable to devise a method by which tryptophan could be estimated quantitatively. A method which naturally suggested itself is the colorimeter, which, as is the case with all similar methods, is very imperfect. We preferred to resort to a method based on the following observation. When bromine water is added to a tryptophan solution a purple coloration develops. The intensity of the color increases with the continued addition of bromine water until a maximum is reached. At this phase the solution becomes very sensitive to further addition of the reagent. As little as one additional drop of bromine water causes the disappearance of the purple color. The nature of the chemical changes, which bring about the described reactions, will be studied in the future.

In applying this property of bromine water in the quantitative estimation of tryptophan, one must bear in mind that there are several other products of protein cleavage which combine with bromine, and some of them form colored substances, the presence of which renders it difficult to distinguish the end point of the tryptophan reaction. In order to obviate this difficulty it was found necessary to titrate, not the solution containing all the products of protein cleavage, but only the fraction precipitated by the reagent of Hopkins and Cole.

<sup>1</sup> *Journ. of Physiol.*, xxvii, p. 418, 1901.

The process of tryptophan estimation in detail is then as follows: The solution of the products of protein digestion or hydrolysis is made to contain 5 per cent. of sulphuric acid and is then treated with the mercuric sulphate reagent<sup>1</sup> of Hopkins and Cole, which yields a precipitate. The reagent is added gradually, the supernatant liquid being tested with bromine water from time to time. It is preferable to end the addition of the bromine just at the point at which the supernatant liquid ceases to form the purple coloration. The mixture is then allowed to stand for twenty-four hours. The precipitate is filtered off, suspended in water containing not more than 1 to 2 per cent. of sulphuric acid, and decomposed by sulphuretted hydrogen. The filtrate from mercuric sulphide is heated on the water bath until all hydrogen sulphide is removed, brought to a definite volume, and is then ready for titration. Fifteen cubic centimeters of the solution are taken in a test tube for analysis. To this are added 2 cubic centimeters of amyl alcohol which serve for the extraction of the coloring matter. The bromine water is added gradually and the tube is shaken vigorously. The addition of bromine water is discontinued as soon as the purple color of the amyl alcoholic layer disappears. Duplicates of the same solution on titration consumed quantities of bromine water differing by from 0.05 to 0.10 cubic centimeter of the reagent.

The concentration of the tryptophan, and the degree of acidity of the solution employed for analysis remain without influence on the end reaction. This can be seen from the following experiments.

I. INFLUENCE OF CONCENTRATION.

Tryptophan solution	10 c.c.	5 c.c.
Water	5 "	10 "
Bromine water	1.85 "	0.9 "

(2 c.c. of amyl alcohol and 6 drops of a 15 per cent. solution of sulphuric acid were added to each tube.)

II. INFLUENCE OF ACIDITY.

Tryptophan solution	10 c.c.	10 c.c.
Water	5 "	5 "
Amyl alcohol	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	—
Sulphuric acid conc.	—	6 drops
Bromine water	1.85 c.c.	1.75 c.c.

<sup>1</sup> Ten per cent. of mercuric sulphate, dissolved in a 5 per cent. solution of sulphuric acid.

Since, however, the precipitate obtained by Hopkins' reagent contains cystin and tyrosin in addition to tryptophan, it was necessary to inquire into the influence of these substances on the titration with bromine. It was found that each of the two substances combines with bromine.

Tyrosin solution	5 c.c.	—
Water	10 "	15 c.c.
Amyl alcohol	2 "	15 "
Sulphuric acid, 15 per cent.	6 drops	6 drops
Bromine water	0.5 c.c.	0.15 c.c.

Titrated until the amyl alcohol layer acquired a pale yellow coloration. Thus 5 cubic centimeters of the tyrosin solution consumed 0.35 cubic centimeter of the bromine water.

The bromine water consumed by a solution containing both tryptophan and tyrosin is equal to the sum of the quantities required for titration of each of the substances separately. This is seen from the following experiment.

Tryptophan solution	10 c.c.	10 c.c.	10 c.c.
Tyrosin	—	2.5 "	5 "
Water	5 "	2.5 "	—
Amyl Alcohol	2 "	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	6 drops	6 drops
Bromine water	1.85 c.c.	2.00 c.c.	2.2 c.c.

Deducting the bromine water required for saturation of the tyrosin one finds that in every one of the three experiments the tryptophan required 1.8 cubic centimeters for its saturation.

It should be remarked, however, that if precautions are taken the quantity of tyrosin present in the tryptophan fraction can be reduced to a mere trace. This is accomplished first by avoiding the addition of an excess of the mercuric sulphate solution. As already mentioned, the addition of the reagent is discontinued as soon as the remaining solution ceases to form the typical coloration with bromine water. The precipitate is to be washed with a 5 per cent. solution of sulphuric acid until the wash water contains no tyrosin. One can calculate the quantity of tryptophan in the presence of tyrosin by making a nitrogen estimation of the solution.

Of greater importance is the presence of cystin as seen from the following experiment.

Tryptophan solution	5 c.c.	5 c.c.	—
Cystin (solution of sodium salt)	—	5 "	5 c.c.
Water	10 "	5 "	10 "
Amyl alcohol	2 "	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	6 drops	6 drops
Bromine water	0.95 c.c.	1.75 c.c.	0.8 c.c.

(The tryptophan solution contained 0.0926 gm. in 100 c.c. of water; of tyrosin 0.069 gm. in 50 c.c.; of cystin also 0.0708 gm. in 100 c.c.)

Since the quantity of cystin can be easily estimated by a sulphur determination there is no difficulty in calculating the quantity of tryptophan present in the solution. As already mentioned the quantity of tyrosin in the tryptophan fraction can be reduced to a mere trace, and since the solution binds but little bromine by its presence, can be disregarded. In estimating the tryptophan one should proceed in the following manner: (1) Titrate the solution of cystin and tryptophan; (2) In an aliquot part of the solution make a sulphur estimation and calculate the quantity of bromine required to saturate it; (3) Deduct the last figure from that obtained on titration of the solution containing both substances and the resulting figure represents the number of cubic centimeters of bromine water required to saturate the tryptophan. It is an advantage to standardize the bromine water with solutions of tryptophan and cystin before each analysis.

A study of the conditions giving the best yield of tryptophan on protein cleavage, and of the quantity of the substance present in various proteids is now in progress.

# Experimental Chronic Nephritis.



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## EXPERIMENTAL CHRONIC NEPHRITIS.\*

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From the slight mention which experimental work on chronic nephritis receives in our text-books and even in more exhaustive monographs on renal diseases it would seem that the result of experimentation conducted with the object in view of reproducing chronic Bright's disease and of elucidating some of the many obscure problems connected with it has been unsatisfactory. Ziegler<sup>1</sup> states very positively:

Die Experimentaluntersuchungen über Nephritis . . . lassen sich für die Pathologie der bei dem Menschen vorkommenden Nephritis nur in sehr beschränktem Maasse verwenden.

Similar remarks may be found in other publications relating to the same subject,<sup>2</sup> and yet a careful review of the literature shows that, although experimental research so far fails to furnish a full explanation of all conditions which in man are associated with this disease, the authors seem to underestimate the importance of what really has been accomplished. The number of instances is not so very small in which experimenters have succeeded in the reproduction of the essential lesion: chronic interstitial nephritis even with marked shrinkage of the kidneys. From our experience in man we must assume that the other disturbances which usually accompany chronic nephritis would have followed in due course had the experiments been continued for a

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\* From the Pathological Laboratory of Cooper Medical College.

1. Ziegler: Lehrbuch, eighth edition, 1895, p. 754.

2. See Lyon: "Inflammatory Changes in the Kidney: An Experimental Study of the Action of Some Toxins and Poisons on the Kidney and also on the Spleen," Jour. Path. and Bact., 1904, ix, 400. Lyon says: In no case and by no variation of the experimental method have I been able to produce and follow the evolution of changes at all analogous to those which we find in sub-acute and chronic diffuse nephritis in man.

sufficient length of time. This, to my mind, is very clearly shown by Ehrlich's<sup>3</sup> and Levaditi's<sup>4</sup> experiments with vinylamin. Vinylamin does not affect the renal cortex, at least when it is used in sufficiently small doses,<sup>5</sup> but produces an extensive necrosis of the papilla. If the animals (mice were used mostly) survive the injection, extensive lesions in the cortex naturally follow which eventually cause considerable contraction. In such animals the usual sequelæ of chronic nephritis: marked edema, hypertrophy of the left ventricle and even retinitis albuminuria were observed repeatedly. This would seem to indicate very plainly that, in whatever way the lesions of the cortex are produced, the other symptoms of chronic Bright's disease follow if there is time enough for their development and if the lesions of the cortex are severe enough. The result of Pässler's and Heincke's experiments<sup>6</sup> is also interesting in this regard. They reduced the renal cortex in dogs very considerably (less than one-half) by operation and prevented the occurrence of the usual compensatory hypertrophy by subsequent operative interference. They found in those animals which survived long enough and which did not develop a cachectic condition a considerable rise in blood pressure followed by hypertrophy of the left ventricle. They did not, however, observe any edema nor symptoms of uremia.

In the following I shall give a brief review of what data I have been able to collect from the literature in regard to the experimental production of chronic nephritis by poisons which directly affect the renal cortex. I shall treat only of those of which it has been reported that they produce interstitial changes.<sup>7</sup> These are aloin,

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3. "Ueber den Zusammenhang von chemischer Constitution und Wirkung," Leyden's Festschrift, 898, p. 647.

4. "Experimentelle Untersuchungen über die Necrose der Nierenpapillæ," Arch. int. de Pharmacodynamie, 1901, viii.

5: Levaditi explains Lindemann's observations to the contrary. Lindemann: "Sur le mode d'action de certains poisons rénaux." (Ann. de l'Inst. Past., 1900, xiv, p. 40) by assuming that he either used too large doses or employed a deteriorated preparation.

6. Verh. der Deutsch. Path. Gesell. Meran, 1905.

7. It has been repeatedly asserted from theoretical considerations that all substances which have an irritative effect on the kidneys should, if employed long enough, cause chronic renal disease with interstitial lesions. Experimentally, there is much to support such a view, still it also would appear from the experimental evidence at hand that certain substances affect the interstitial tissue more strongly than others. It is these that we treat of here.



boracic acid, cantharidin, potassium chlorate, chromic acid and the chromates, lead and other heavy metals, oxalic acid and oxamid, sulphuric acid and various bacterial toxins.

*Aloin.*—The effects of aloin on the kidneys were studied by Kohn<sup>8</sup> in 1882. Microscopically he found degenerative processes and necroses in the epithelium, but no interstitial changes. Mürset<sup>9</sup> had very similar results, but in addition found areas of cellular infiltration; in one case even beginning atrophy of the tubules as a result of the thickening of the connective tissue.

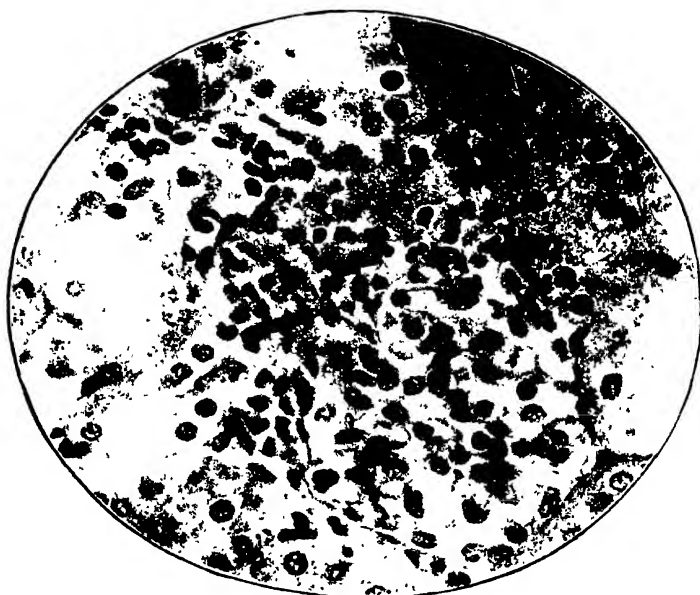


Fig 1. Beginning interstitial inflammation and invasion of glomerulus in guinea-pig No. 2. Flemming, Saffraun. High power.

*Borax.*—It has lately been maintained by Harrington<sup>10</sup> that by feeding cats for some time (133 days) food containing considerable doses of borax he had produced marked renal lesions not confined to the epithelium, but involving the interstitial tissue extensively. His results,

8. "Beitrag zur Wirkung der Aloë," Berlin klin. Woch., 1882, xix, p. 68.

9. "Untersuchungen über Intoxicationsnephritis," Arch. f. Path. u. Pharm., 1885, xix, p. 310.

10. "Borated Food as a Cause of Lesions of the Kidneys," Am. Jour. Med. Sci., 1904, p. 418.

if confirmed, should have considerable influence in shaping pure-food legislation.

*Cantharidin*.—Cantharidin has been used very much more extensively<sup>11</sup> in experimental work of this kind. The principal renal lesion in this form of poisoning is a glomerulo-nephritis which has been carefully studied by Cornil,<sup>12</sup> Eliaschoff<sup>13</sup> and Welch.<sup>14</sup> In 1882 Aufrecht<sup>15</sup> observed a typical contracted kidney with marked macroscopic lesions in a rabbit (experiment No. 8) that he had given 25 subcutaneous injections of cantharidin in the course of 4 months. Of the microscopic picture he says:

Interstitialien ausserordentlich stark verbreitert, in denselben zahlreiche grosse ovale und rundliche Kerne.

Another rabbit, however (experiment No. 9), treated similarly for 3 months did not show any lesions in the interstitial tissue. Similarly varying results were obtained by Germont.<sup>16</sup> In one of his guinea-pigs he found interstitial lesions, although they were not so marked as in Aufrecht's case; in a rabbit they were absent. Lyon<sup>2</sup> also used cantharidin for part of his experimental work. He failed to produce chronic progressive lesions, still in some instances he saw areas of cellular infiltration around veins.

*Potassium Chlorate*.—The most important change observed in acute and chronic potassium chlorate poisoning, as is well known, is a marked decomposition of the blood with the production of methemoglobin and intense pigmentation of the kidneys. There is more or less degeneration of the epithelium. In a case of this form of poisoning in man with death 12 days after ingestion of

11. Only those papers which have a direct bearing on the question under discussion are mentioned.

12. Jour. de l'Anat. de Robin, 1880; also Cornil and Brault: "Etudes sur la Pathologie du Rein," Paris, 1884.

13. "Ueber die Wirkung des Cantharidin auf die Nieren," Virch. Arch., 1883, xciv, 323.

14. "Experimental Study of Glomerulo-Nephritis," THE JOURNAL A. M. A., 1886, vii, p. 49.

15. "Die Schrumpfnieren nach Cantharidin," Centrbl. f. die med. Wiss., 1882, xx, 849; also "Die Nephritis nach Cantharidin," Path. Mitt., ii, 1883, p. 19.

16. "Contribution a l'Etude Expérimentale des Nephrites," Thèse de Paris, 1883.

the poison Marchand<sup>17</sup> found small areas of cellular infiltration in the kidneys.

*Chromic Acid.*—Striking results were obtained from the very first with chromic acid and its salts. Soon after Gergens<sup>18</sup> had discovered that the absorption of this substance caused a very marked acute nephritis, its effects on the kidneys were very carefully studied by Kabierske,<sup>19</sup> who in chronic intoxications found cellular infiltration around the blood vessels after the sixth day and beginning proliferation of the cells of the interstitial tissue. Repeated subcutaneous injections of small doses were followed by marked thickening of the interstitial connective tissue.<sup>20</sup> Kabierske's results were confirmed by Pander,<sup>21</sup> who found that in his experiments the interstitial infiltration began in the second week and was marked after 3 to 4 weeks. In 1892 von Kahlden<sup>22</sup> made a few experiments with chromic acid on rabbits and dogs, the longest one lasting 14 days only. Naturally he was unable to demonstrate any interstitial lesions. Similarly Burmeister,<sup>23</sup> confining his researches to the more acute stages, found only a very moderate proliferation of the interstitial tissue.

*Lead.*—The literature on experimental lead poisoning is very large. Many papers, however, do not refer to the renal complications or if they do so only incidentally. I mention only those papers which are most important in regard to the renal lesions. That chronic nephritis is a frequent complication in chronic lead poisoning in man and also that albuminuria and renal lesions can be produced in animals by the introduction of lead in various forms has long been recognized. In experimental lead poisoning, as in other intoxicants, the occurrence of parenchymatous lesions in the kidneys was first observed

17. "Ueber Intoxication durch chlorsaure Salze," Virch. Arch., 1879, lxxvii, p. 455.

18. "Ueber die toxische Wirkung der Chromsäure," Arch. f. exp. Path. u. Pharm., 1876, vi, p. 148.

19. "Die Chromniere," Diss., Breslau, 1880.

20. As the lesions in acute poisoning and the initial lesions in chronic poisoning were entirely epithelial, Weigert, who studied Kabierske's specimens, regarded his work as an experimental confirmation of his views of the primary character of the epithelial lesions in chronic nephritis in man.

21. "Arbeiten des pharmak.," Instituts zur Dorpat, II, p. 1.

22. "Die Aetiologie und Genese der akuten Nephritis," Ziegler's Beitr., 1892, xi, p. 441.

23. "Beitr. zur Histogenese der acuten Nierenentzündungen," Virch. Arch., 1894, cxxvii, p. 405.

(by Cornil, for instance, as early as 1863). In 1880 Raimondi<sup>24</sup> found in guinea-pigs that had been given lead for some time signs of beginning chronic nephritis, and soon afterward Charcot and Gombault<sup>25</sup> published their very important contribution to the subject of chronic experimental nephritis. They mixed with bran, which was part of the feed of their guinea-pigs, very small doses of carbonate of lead. From time to time they stopped the administration of lead entirely. In this way they succeeded in keeping some of their animals alive for a con-

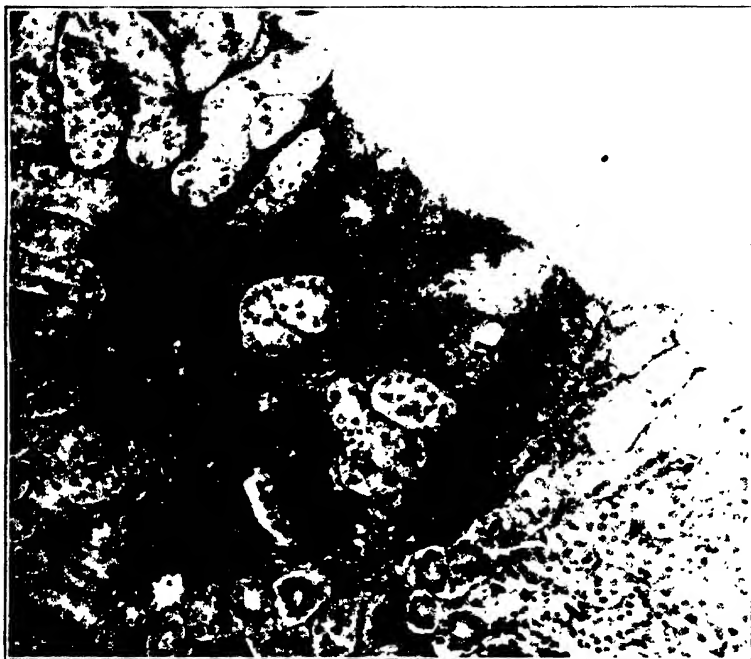


Fig. 2.—Section of renal cortex of guinea-pig No. 5, showing contracted area of chronic nephritis. Low power. van Gieson.

siderable time (3 months to 1 year), and in them observed renal lesions which certainly resemble those found in chronic nephritis in man very closely. With their paper they give very convincing plates showing the granular appearance of the kidneys on the surface and pic-

24. "Degli avvelenamento lenti di arsenico, mercurio e piombo," etc., *Ann univ. de Med.*, 1880, sixty-sixth year, cell, 52.

25. "Note relative à l'étude anatomique de la nephrite saturnine expérimentale," *Arch. de Phys.*, 1881, second series, viii, 126.

tures of sections with marked interstitial changes and the usual distribution. Even if we do not agree entirely with the theoretical deductions which they draw from these experiments, namely, that the condition is what they term a *cirrhose épithéliale*, that is, that the epithelial changes are primary, we must still acknowledge the great value of their investigation in demonstrating in the first place the possibility of the experimental production of very definite chronic nephritis and also the direct relation of lead to this process. It is of some interest to note that in none of their experiments they were able to detect any albumin in the urine of the diseased guinea-pigs. Hoffa<sup>26</sup> had similar, although not so marked results, with lead acetate in rabbits. He also noted an early hyperplasia of the connective tissue. In 1883 Ellenberger and Hofmeister,<sup>27</sup> in studying the effect of lead in ruminants, found a diffuse nephritis in sheep. Coen and d'Ajutolo's<sup>28</sup> investigations are confirmatory in so far as they observed areas of cellular infiltration in the kidneys of rabbits which had been under the influence of lead given in small doses for considerable time, to 155 days, but they failed to produce any typical contracted kidneys. They remark, however:

Es ist höchstwahrscheinlich, dass bei längerer Einwirkung des Giftes auch bei unseren Versuchstieren sich eine indurative Atrophie, eine echte Nierencirrhose würde herausgebildet haben.

Prévost and Binet<sup>29</sup> did not get any striking results in guinea-pigs with lead acetate, but of their series of experiments with carbonate of lead, also given to guinea-pigs, some of which survived for a little more than a year, they say:

Lésions rénales très accentuées chez la plupart d'entre eux, surtout quand l'intoxication avait duré longtemps.

Rats treated in the same way showed similar lesions. Oliver<sup>30</sup> and Stieglitz<sup>31</sup> were not so successful, probably

26. Ueber Nephritis saturnina., Diss. Friburg, 1883.

27. "Zur physiol. Wirkung und Deposition der Bleisalze bei Wiederkäuern," Ber. f. d. Veterinärwesen in Sachsen, 1883, II.

28. "Sulle alterazioni istol. del reni . . . nell' avvelenamento cronico da piombo," Ziegler's Beitr., 1888, VII, p. 480.

29. "Recherches exp. sur l'intoxication saturnine," Rev. de la Suisse Romande, 1889, IX, pp. 606, 669.

30. "Lead Poisoning in Its Acute and Chronic Manifestations," Lancet, 1898, LIX, I, pp. 530, 588, 644.

31. "Eine experimentelle Untersuchung über Bleivergiftung," etc., Arch. f. Psych. und Nervenkrank, XLIV, 1892, p. 1.

because their animals did not survive so long; still they noticed areas of cellular infiltration in the later stages. Annino<sup>32</sup> did not observe any interstitial changes, but Hirsch<sup>33</sup> and Paviot<sup>34</sup> again called attention to the occurrence of a productive interstitial nephritis. It is remarkable that the two latest papers on this subject should be so much at variance with former observations. Oppenheim,<sup>35</sup> who experimented on rabbits with subcutaneous injections of lead acetate in small doses, did not notice any changes in the kidneys. His work, however, is largely chemical and does not contain any records of microscopic investigation. Jores,<sup>36</sup> on the contrary, made a very careful microscopic study of the kidneys in his cases. Although all his animals (rabbits) lived for over two months and one of them for 14 months and 20 days, he remarks:

Die interstitiellen Processe kommen beim Kaninchen erst sehr spät zur Ausbildung. Von geringen Herden abgesehen, bin ich Wucherungen des Bindegewebes nur einmal begegnet, bei dem Tier welches die längste Untersuchungsdauer hat. Auch hier lag keine der menschlichen Schrumpfniere analogen Verbreiterung des wuchernden Bindegewebes vor, sondern der Process beschränkte sich auf einen narbigen Herd in einer Niere.

*Heavy Metals.*—In regard to the influence of heavy metals, in general, on the kidneys, some remarks of Kobert<sup>37</sup> on what he calls "die Metallniere" are very suggestive. He showed that mangan, iron, nickel and cobalt which are very poorly absorbed from the intestines, produce a marked acute nephritis on subcutaneous injection. If the administration is discontinued the process heals promptly. In a case of chronic experimental mangan poisoning, however, he observed a more chronic form of nephritis with new formation of connective tissue and even secondary contraction.

*Mercury.*—Although acute mercury poisoning may give rise to a very marked acute nephritis with necrosis

32. "Avvelenamento cronico da piombo," Arch. Ital. di Clin. Med., xxxii, Rev. in Virchow Hirsch Jahresberichte, 1894.

33. "Experimentelle Untersuchungen zur Lehre von der Bleinniere," J. D., Leipzig, 1891.

34. "Pathogénie des lésions rénales dans le saturnisme," Gaz. hebdomad., 1886, new series, i, 544.

35. "Zur Kenntniss der experimentellen Bleivergiftung," J. D., Berlin, 1898.

36. "Ueber die pathologische Anatomie der chronischen Bleivergiftung des Kaninchen," Ziegler's Beitr., xxxi, 1902, p. 183.

37. "Zur Pharmakologie des Mangans und Eisens," Arch. f. experim. Path., 1883, xvi, 301.

and calcification of the epithelium in man and in the lower animals, it apparently does not lend itself very well to the production of any lesions resembling those found in chronic Bright's disease in man. This was at least Lyon's<sup>2</sup> experience. He reports very slight, if any, interstitial changes.

*Oxalic Acid.*—Oxalic acid, besides being deposited in the kidneys in large quantities, is known to cause considerable renal irritation, slight epithelial lesions, but ap-

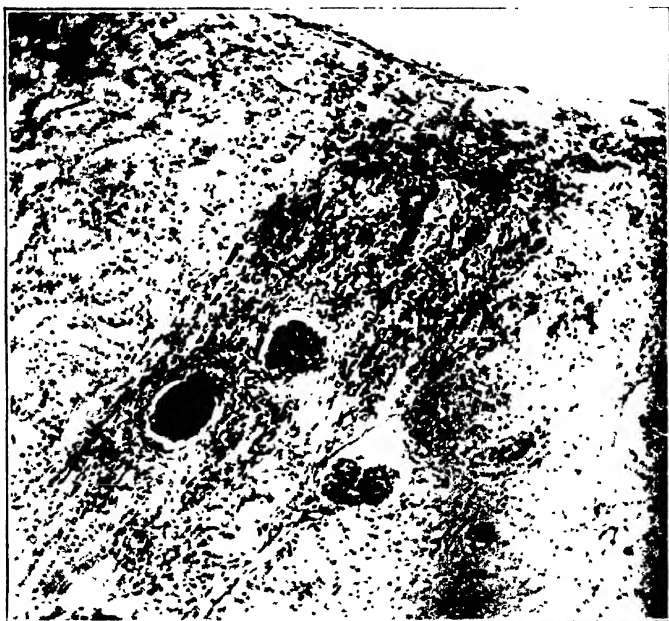


Fig. 3.—Subcortical area of chronic nephritis in dog No. 1. Low power. van Gieson.

parently no effect on the interstitial tissue. It is somewhat surprising, therefore, that Ebstein and Nicolaier<sup>38</sup> announced at the Congress for Internal Medicine at Wiesbaden, in 1892, that they had succeeded in producing typical contracted kidneys in two dogs by feeding them small doses of oxalic acid and oxamide for 169 and 304 days, respectively. I have not been able to find

38. "Ueber experimentelle Erzeugung von Schumpfnieren durch Oxalsäure-Oxamidfütterung," Verh. des Cong. f. inn. Med., Wiesbaden, 1892, xl, p. 318.

their more extensive publication which they announce, nor have their experiments been confirmed so far as I know, which would be very desirable, as the number of them is so small and as it is well known that dogs not so infrequently suffer from spontaneous chronic nephritis.

*Sulphuric Acid.*—In regard to the effect of sulphuric acid on the kidneys, I should like to confine myself to a short reference to Fränkel and Reiche's<sup>39</sup> paper which shows that in this form of poisoning degenerative changes in the epithelium are common and slight interstitial changes may be observed occasionally.

*Bacterial Toxins.*—As acute infectious diseases are frequently followed by acute nephritis which fortunately usually heals—completely so far as we know—but which may occasionally terminate in chronic nephritis (in cases of scarlet fever, for instance) the experimental study of the effect of bacterial toxins on the kidneys has suggested itself to many authors. Pernice and Scagliosi<sup>40</sup> describe the occurrence of glomerulitis and consecutive nephritis in animals following injections of living bacterial cultures and bacterial toxins. According to their idea, the process begins in the circulatory apparatus with endarteritis, followed by disturbances in circulation and hemorrhages. Later, changes occur in the Malpighian bodies and the epithelium of the tubules. In still later stages there may be collapse of the tubules, simulating thickening of the interstitial tissue. Morse,<sup>41</sup> under Councilman's direction, made similar experiments. In only 3 out of a number of experiments he found areas of cellular infiltration. He argues:

It must be admitted that the human kidney is more or less constantly exposed to the action of chemical substances produced by bacteria. Hence it would seem justifiable to assume that a certain proportion of the cases of chronic nephritis in man may be due to the action of such substances.

Lyon,<sup>2</sup> in experimenting with diphtheria toxin, observed hemorrhages, degeneration of the epithelium, later areas of cellular infiltration around the blood vessels, but, although he continued the injections for months, he

39. "Ueber Nierenveränderungen nach Schwefelsäurevergiftung," Virch. Arch., 1893, cxxxi, p. 130.

40. Beitrag zur Aetiologie der Nephritis (Experimentelle Nephritis von bakterischem Ursprung), Virch. Arch., 1894, cxxviii, 521.

41. "Changes Produced in the Kidneys by the Toxins of the *Staphylococcus pyogenes aureus*," Jour. Exp. Med., 1896, i, p. 613.



could not produce any fibrous change. Other investigators, however, describe much more marked lesions in animals which had been under the influence of this substance. It would lead us too far to attempt a complete review of the literature<sup>42</sup> of this part of our subject. Suffice it to say that, however interesting these experiments may be and however plainly they may show that bacterial toxins may produce chronic renal disease, the enthusiastic belief of some that all cases of chronic Bright's disease in man or even the majority should be explained on this basis seems hardly warranted. All experimental data show plainly that in order to produce chronic nephritis the action of the poison on the kidneys must be long continued, otherwise the lesions heal, and if considerable tissue is lost it is made up for by compensatory hypertrophy. As in most infectious diseases in case of survival the infectious agent which furnishes the poison is completely destroyed after a while, the production of toxin must necessarily stop and with that the possibility of the development of a progressive chronic renal disease. All that may remain is a certain vulnerability of the kidneys, but even this occurrence has never been proved absolutely.

#### AUTHOR'S EXPERIMENTS.

For my own experiments I first selected lead because it is the only substance of which we know with any degree of certainty that it produces chronic nephritis in man. In all my experiments I gave the poison by mouth because this mode of administration came closer to what occurred under natural conditions. In an attempt at reproducing a disease of such chronicity the smallest possible doses had, of course, to be employed; not too frequently, in order not to kill the animals prematurely nor even to interfere too much with their general nutrition. In the case of lead it was easy to determine by blood examination whether the doses were large enough to produce any appreciable effect. Even after the very small doses which were employed the well-known lead anemia promptly developed. As it seemed that after a while the animals became accustomed to the metal, to a certain extent, the doses were slowly increased; still they

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42. In regard to this see Asch, P.: "Ueber den Einfluss der bacteriellen Stoffwechselproducte auf die Niere," Strassburg, 1904, published by L. Beust.

were never very high. The severe changes which resulted from the administration of a few grams of lead in the course of a year or so are certainly a renewed proof of the extreme toxicity of this metal and a renewed strong argument in favor of very strict protective legislation with the object in view to prevent not only accidental lead poisoning, but also the introduction with the food of any substances which might have an irritating effect on the kidneys. The anemia after a while assumed the character of a pernicious anemia with marked decrease in



Fig. 4.—Glomerulo-nephritis in dog No. 3. van Gieson. High power.

the number of erythrocytes, with the appearance of many macrocytes and microcytes and some poikilocytes in the peripheral circulation. The nucleated red blood corpuscles were very numerous and many of them were of the megaloblastic type. At autopsy the bone marrow resembled that found in pernicious anemia macroscopically. It contained an immense number of nucleated red cells, among them many megaloblasts. The liver and especially the spleen and the kidneys also were full of hemosiderin. The changes were very much like those which

can be experimentally produced by toluylendiamin. This extreme anemia was the greatest obstacle encountered in the attempt at continuing the experiments for the desired length of time.

#### RESULTS OF EXPERIMENTS ON GUINEA-PIGS.

The following is a short record of the result of these experiments. They were conducted according to the method of Charcot and Gombault. Small doses of carbonate of lead were mixed with bran. Guinea-pigs were fed on greens and the poisoned bran on alternating days. Two guinea-pigs were in one cage. In the beginning of the experiments 1 grain of carbonate of lead was put into the bran, later 2 and 3 grains. An exact dosage is, of course, impossible in this way. The experiment was started Dec. 10, 1905, with 6 guinea-pigs. Four of these guinea-pigs have so far been examined, one after 2 months, two after 3½ months and one after about 5 months, having taken approximately 1½ to 3 grams of carbonate of lead. The lesions were very similar in all four animals.

Macroscopically the kidneys were a little pale and opaque, otherwise unaltered. Sections of pieces hardened in Flemming's solution show comparatively slight changes in the epithelium of the convoluted tubules and the ascending loops of Henle. Some of the cells show granular degeneration and also slight fatty changes. There are a few cells with pyknosis of the nuclei. Some of the epithelial cells have become detached and have dropped into the lumen of the uriniferous tubules. In some of the specimens one finds collections of such cells in the large ducts in the pyramids, the epithelial covering of which is unaltered. An active regenerative process in the epithelium is indicated by the occasional occurrence of nuclear figures. In all sections there are small areas of cellular infiltration around the blood vessels at the hilum of the glomeruli. These cells are mostly large cells of epithelioid type with large vesicular nuclei. Occasionally mitoses can be seen in the nuclei of these cells. One also finds a few lymphocytes and cells resembling plasma-cells. Similar cells have accumulated in moderate number along the course of the vascular loops of the glomeruli near the hilum. The larger blood vessels are normal. In some places these collections of cells crowd on the adjoining tubules which then show more or less

atrophy, but no more nor less degenerative changes in the epithelium than in other parts (Fig. 1.). The most important other organs were examined microscopically, but no lesions were present.

#### EXPERIMENTS ON DOGS.

**EXPERIMENT 1.**—Cocker, female, received every fourth or fifth day, starting from Oct. 18, 1904, a certain quantity of 1/1,000 solution of lead acetate with the drinking water. During October, November and December the dose was 5 c.cm.; in January, 1905, 10 c.cm.; in February and part of March, 20 c.cm.; the rest of March, 30 and 40 c.cm.; in April and May, 55 c.cm.; in June, at first 100, later 150 c.cm.; in July, 150; in August, September and October, 100; in November and December and January, 1906, again 50 c.cm. Dog died Jan. 5, 1906. This dog received in all, 8.65 gm. of acetate of lead in the course of fifteen months. The urine was examined at least once a week with the ordinary clinical tests, and remained normal all the time. The blood showed the usual progressive anemia.

*Autopsy.*—At autopsy the kidneys were found hard and somewhat cyanotic. In the cortex there were a considerable number of small but plainly visible retracted scars, mostly at the poles. There was no deposit in the pyramids. Joints were normal. In lower part of abdominal aorta a slight irregular fibrous thickening was present in the intima which on microscopic examination did not show any cellular infiltration or any degenerative changes. It apparently was an old accidental non-progressive lesion. So far as one can judge there was no marked enlargement of the heart,<sup>43</sup> the right ventricle was perhaps slightly dilated, but although the liver was cyanotic there was no evidence of cyanotic atrophy.

*Microscopic Examination.*—The following microscopic changes were found in the kidneys.

**Epithelium:** The functioning epithelium in the convoluted tubules and in the ascending loops of Henle showed rather diffusely various stages of degeneration (granular and fatty change of a mild degree). Some of the nuclei were pyknotic, other cells did not show any nuclear staining. A certain number of epithelial cells had become detached and had dropped into the lumen. Such desquamated cells were also found in the large ducts in the pyramids which were otherwise normal. Most epithelial cells contained much brown granular pigment.

Practically all glomeruli were diseased and very similarly affected. There was a slight swelling and partial desquama-

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43. It is very difficult to form an opinion as to what size the heart should be in a given dog as its size is evidently not directly proportionate to the size and weight of the dog and as we have no other standards to compare it with, such as the fist in man.

tion of the capsular epithelium. The capsules contained a little granular material, and a few red blood corpuscles in places. The vas afferens and the parts of the capillaries near it showed a marked thickening of the wall without much obstruction of the lumen. The thickening was partly due to an increase in the number of cells composing the wall, the new cells being rather large with vesicular nuclei, partly to the presence on the outside of the normal capillary wall of an apparently hyaline substance which stains brown with the van Gieson method. In some places the new formation in this hyaline substance of very delicate connective tissue fibrillæ

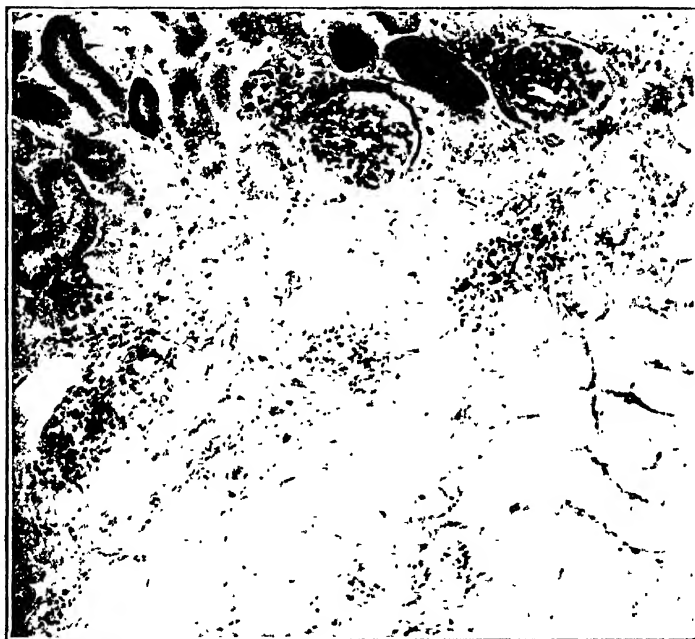


Fig. 5.—Beginning Interstitial Inflammation in bichromate dog Flemming, Safranin. Low power.

which stain purple with the van Gieson method was evident. The process represented evidently a mild degree of chronic glomerulo nephritis (Fig. 4).

The interstitial tissue in every section showed several large more or less cone-shaped areas of fibrous thickening, with considerable cellular infiltration in places. These areas were situated along the course of the blood vessels, irregularly scattered in the cortex, but most numerous in the outer portion, and also near the dividing line between cortex and medulla. The tubules in these areas show a marked atrophy. Few of the

atrophic tubules and also a few others contained hyaline casts, which, however, were scarce (Fig. 3).

**EXPERIMENT 2.**—Old pug dog, male, started from Oct. 19, 1904, receiving 1/1,000 lead acetate solution with his drinking water every fourth or fifth day very much in the same way as dog in Experiment 1. He took in all 8.42 gm. of lead acetate in fifteen months. The urine was examined at least once a week with the ordinary clinical tests and did not show anything abnormal at any time except on December 19, when, after a severe exertion (a fight with another dog), it contained much albumin and many hyaline and granular casts. The blood showed the usual progressive anemia. The animal died Jan. 19, 1906.

**Autopsy.**—At autopsy the kidneys were a little hard, cyanotic, but otherwise of normal appearance. No deposits in the pyramids. Aorta and joints were normal. The heart seemed to show a moderate dilatation on both sides. The liver showed a marked passive congestion, but microscopically there was no evidence of cyanotic atrophy. Microscopic examination of the kidneys revealed the same lesions as in dog in Experiment 1, except that the areas of chronic interstitial nephritis, although just as numerous, were not quite so large.

**EXPERIMENT 3.**—Small bitch. Experiment started Oct. 25, 1905. Mode of administration was the same as in Experiments 1 and 2. The dog took in all 7.28 gm. of acetate of lead in the course of twelve months. The urine was examined as usual and found normal at all times. She was examined Oct. 26, 1905.

**Autopsy.**—At autopsy the kidneys were hard, the surface smooth, the markings not so distinct as normally, and the cortex a little opaque, especially near the medulla. There were no deposits in the pyramids. Joints and aorta were normal. The heart seemed to show a moderate dilatation on both sides. There was no cyanotic atrophy of the liver. The beginning of the aorta showed a diffuse fibrous thickening of the wall to about twice the normal diameter. There was similar but not so marked thickening of the arteries of the neck. (Microscopically there was much dense hyaline fibrous tissue in adventitia and along the vasa vasorum. There was no cellular infiltration.) There was some dead degenerated fetus *in utero*. The microscopic lesions in the kidney were very much like those in dogs in Experiments 1 and 2. The areas of chronic interstitial nephritis were somewhat smaller, more cellular, situated almost exclusively at the dividing line between cortex and medulla. Some of them reached a distance into the medulla. The thickening of the walls of the vascular loops of the glomeruli near the hilum was entirely cellular (Fig. 4).

**EXPERIMENT 4.**—Experiment started Jan. 30, 1906. Mode of administration was the same as in the other dogs. This dog took in all only 0.85 gm. of acetate of lead in one and one-

half months. He was killed by another dog March 4, 1906. The urine was examined as usual and found normal.

**Autopsy.**—At autopsy no macroscopic lesions were found in the kidneys. Sections showed granular and beginning fatty change in some of the epithelial cells in the convoluted tubules and the ascending loops of Henle, beginning desquamation of the epithelium in a few places. In serial sections a few small areas of cellular infiltration around the blood vessels near glomeruli were found. The cells were partly lymphocytes, partly larger cells with vesicular nuclei. They began to crowd the adjoining tubules and extend into the glomeruli along their stalks.

The kidneys of all dogs that were treated a sufficient length of time showed a marked chronic interstitial nephritis. In the dog in Experiment 1 the change was plainly visible to the naked eye. Although the condition is not entirely that of an extreme granular atrophy, the lesions are certainly definite enough to make sure of the nature of the process. It is interesting to note that, in spite of careful repeated examination neither albumin nor casts were ever found in the urine of these dogs. It is true the urine was not directly obtained from the animals, but collected in metabolism-cages. It is possible, therefore, that a few casts might have escaped observation and I am fairly certain that, under more favorable conditions, renal epithelium could have been detected in the sediment. Albumin in appreciable quantity was certainly absent, as it was also in the experiments of Charcot and others. This observation gives support to the clinical suspicion that in man also such lesions may develop with very little, if any, change in the urine. It also corresponds with our clinical experience that in dog in Experiment 2 a severe exertion was followed by the appearance of albumin and casts in the urine.

The experiments on the guinea pigs and on dog 4 reveal the initial stages of the process. The lead produces a certain amount of degeneration, some necrosis and desquamation in the epithelial cells of the convoluted tubules and ascending loops of Henle. One also observes evidence of a probably regenerative proliferation in them. Casts were present, but not very numerous.

The cellular infiltration commences around the blood vessels near the glomeruli. Part of the cells which collect in the tissues are lymphocytes, the larger part, however, probably proliferated connective tissue cells. That

at least some of them are formed *in situ* is shown by the occurrence of mitoses. Considering the chronicity of the process, one would naturally not expect to see many karyokineses, even though most of the cells were the results of proliferation of the fixed cells of the tissue. On account of their scarcity, the mitoses have not been mentioned by all observers (Burmeister,<sup>23</sup> for instance, claims that none were present in his sections). In the end the formation of quite a little new fibrous tissue may be the result of this proliferation of the connective tissue cells.

Similar cells make their appearance along the vascular loops of the glomeruli at their point of attachment to the adjoining tissue and ultimately lead to the development of a chronic glomerulo-nephritis.

Most observers noting the early degenerative changes in the epithelium and the much later occurrence of the lesions in the interstitial tissue have followed Weigert's lead in contending that the epithelial changes are primary and those in the connective tissue secondary in character. I confess I can not see the force of the argument. If any irritant acts simultaneously on both the epithelium and the connective tissue, this must necessarily happen. The epithelial changes are early because the degeneration can and does occur within a short time, whereas the more slowly developing changes in the connective tissue show much later, necessarily. Moreover, as has been pointed out by former observers (Germont,<sup>16</sup> Stieglitz,<sup>31</sup> Paviot<sup>34</sup> and others), there is no direct topical relation between the degenerative changes in the epithelium and the proliferative changes in the connective tissue. The epithelial degeneration is more or less diffuse, the connective tissue changes localized and the tubules at the point of the lesion in the connective tissue do not show a particularly severe degree of degeneration. Moreover, it is plainly brought out by all experimental evidence on this question that the more chronic the process the less marked the lesions in the epithelium and the more pronounced the interstitial changes. If the interstitial changes depended directly on the epithelial destruction such could not be the case. The probability, then, would be that large doses would be followed by very severe epithelial lesions with rapid fatal termination, medium repeated doses would cause still severe but not immediately fatal epithelial lesions followed by inter-



stitial lesions and small repeated doses would give very slight epithelial lesions not sufficiently serious to give rise to secondary changes in the interstitial tissue. This is not borne out by experiments with any of the substances which so far have been investigated. I believe, therefore, that, on the contrary, the experimental evidence furnishes almost positive proof that the changes in the epithelium and in the connective tissue are co-ordinate, not subordinate, to one another. They are the effect of the same cause acting simultaneously on different tissues, with different vulnerability and different reaction time, so far as visible changes are concerned.

On account of the difficulties encountered in keeping the animals alive for the necessary length of time and also in order to supplement the experiments made with lead, two dogs were given small doses of bichromate of potash in milk. Both were treated exactly in the same manner. One of the dogs was unfortunately lost in the confusion following the earthquake in San Francisco. In the other one, which was examined some time before, the following condition was found at autopsy:

*Experiment with Bichromate of Potash with Milk.*—Experiment started Nov. 10, 1905. The dog was given 1/50 aqueous solution of bichromate of potash in milk every fourth or fifth day. The initial dose was 5 c.cm., which was later slowly increased to 20 c.cm. He took in all nearly 11 gm. of bichromate of potash in four and one-half months. On March 22, 1906, the dog was chloroformed and examined. During the course of the experiment some anemia developed with a few normoblasts in the peripheral circulation, and very many in the bone-marrow, but the anemia was very mild compared with that observed in the animals taking lead. No megaloblasts were found. The urine was examined once a week with the ordinary clinical methods. No albumin or casts were demonstrated except perhaps a trace of albumin on January 24, and again on February 28. In a fresh sample of urine obtained directly from the dog on February 14, a few cells resembling renal epithelium were found in the sediment. The dog vomited repeatedly after having taken the bichromate and at autopsy considerable gastritis and a marked follicular enteritis were noted, especially in the duodenum and upper part of the small intestine. There were, however, no ulcers nor even any epithelial defects to be found microscopically in sections. The heart was rather large. The surface of both kidneys was slightly irregular and numerous badly defined hard grayish white areas could be discerned in the cortex. Sections of specimens hardened in Flemming's solution showed the following lesions: These were granular and beginning fatty degeneration of many epithelial cells in

the functioning tubules. Some of the cells were necrotic, but still *in situ*, others had become detached. No mitoses were found in the epithelial cells. There were no casts. Many glomeruli showed a marked thickening of the tunica propria of the capsule. The capsular spaces of many of them were dilated and filled with coagulated fluid, sometimes containing a few red blood corpuscles.

Throughout the cortex there were a large number of areas of cellular infiltration of considerable size. The larger ones showed considerable new formation of young fibrous tissue in the center. These areas were always perivascular, usually near glomeruli. In many places cells similar to those found in these areas were also present alongside the vascular loops of the glomeruli near the hilum. The cells were partly lymphocytes, frequently, however, larger cells with large vesicular nuclei which were often more or less distorted. Nuclear figures could be found in some of these cells. In all foci a few very large cells with large protoplasmic bodies filled with coarsely granular materials were encountered, the granules staining dark brown or almost black with osmic acid. The larger arteries were surrounded by thick masses of old hyaline fibrous tissue, but there was no endarteritis (Fig. 5).

Sections of the liver showed much fat in the liver cells and also some in many endothelial cells of the capillaries. In a few places there was a slight cellular infiltration of the periportal connective tissue.

The experiment shows that, in spite of the fact that this dog took the poison for  $4\frac{1}{2}$  months only, the lesions in the kidneys were more marked than those in the lead dogs that had been under observation for a year and longer. The bichromate of potash, therefore, seems to promise for future experimentation along these lines. Although the lesions were so much more severe, the urine in these dogs nevertheless did not contain at any time any appreciable amount of albumin. Only once cells were discovered in the sediment which looked like renal epithelium.

The experiments will be continued in the hope of obtaining still more marked lesions of the kidneys, of producing more definite disturbances in circulation with consecutive hypertrophy of the heart, and of obtaining some information about the relation of renal and vascular disease in chronic nephritis. In none of the experiments so far has any definite arteriosclerosis been present in the affected kidneys or in any other part of the vascular system except some evidently old accidental lesions.

I believe these objects can be accomplished by still more cautious administration of the poison and by con-

tinuing the experiments still longer than has been done heretofore.

Publication of my observations at this early stage may seem to need some justification. I hope, however, that it may contribute to dispel erroneous conceptions in regard to the value of this work, especially in reference to human pathology and to encourage others, with perhaps better facilities at their disposal, to undertake investigations along the same lines.

#### ADDITIONAL NOTE.

Since the foregoing was written guinea-pig No. 5 died on Sept. 7, 1906. It had received the same treatment as guinea-pig No. 4. It had not taken any more lead since May 10, 1906, except a few small doses in August. Postmortem examination showed in both kidneys very many just visible retracted scars in the cortex underneath the capsule. They were placed about equal distances from another. There were no adhesions between cortex and capsule.

On microscopic investigation they proved to be areas of new formed fibrous tissue with considerable cellular infiltration. Most of the tubules in these areas show a marked atrophy. There is little fat in the epithelium of the atrophic tubules. The epithelium in all other tubules does not show any lesions either in ordinary specimens nor in those prepared with the Flemming mixture (Fig. 2).

In concluding, I wish to acknowledge my indebtedness to the Rockefeller Institute for Medical Research for a grant of money which has enabled me to do the experimental work reported on in this paper.

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29 (172)

**Secondary peristalsis of the esophagus — a demonstration on  
a dog with a permanent esophageal fistula.**

By **S. J. MELTZER.**

[*From the Rockefeller Institute for Medical Research.*]

The peristalsis of the esophagus with which every one is familiar is that which follows an act of deglutition. About a year ago I reported to this society that experiments which I had made on rabbits demonstrated that the esophagus is capable of peristaltic movements not initiated by deglutitions. Injections of indifferent solutions or of air directly into the esophagus cause there a regular peristaltic movement. This latter form of peristaltic movement, which for the sake of brevity I shall henceforth term *secondary peristalsis*, differs from the *primary peristalsis*, the one which follows deglutition, essentially through the nervous mechanism by which it is controlled. All the movements of the complicated act of deglutition are managed by a reflex mechanism, with only one sensory stimulus for its initiation and a series of consecutive motor impulses going to every part of the long path of deglutition; it is practically a single reflex. The reflex mechanism of the secondary peristalsis, on the other hand, consists of a chain of reflexes; each part of the esophagus sends up to the center a sensory impulse started by the presence of the bolus in that part and receives in turn a motor impulse. The secondary peristalsis therefore requires the presence of some sort of a bolus within the esophagus and presupposes the integrity of the latter; whereas the primary peristalsis requires neither a bolus nor the integrity of the esophagus;

even if a large section of the latter is removed, the peristalsis appears in the lower segment in due time after each deglutition as long as the vagus nerves remain intact.

Recently secondary peristalsis was studied in the esophagus of dogs, in which animals it appeared promptly and was easily demonstrable. The bolus consisted mostly of a piece of absorbent cotton attached to the middle of a long thread, one end of which ran through an opening in the floor of the mouth and the other through an opening in the stomach. The animal was of course narcotized but anesthesia interferes greatly with both forms of peristalsis. The observations however were made when the animal recovered from the anesthesia. It was found that the bolus went down to the stomach from any part of the esophagus without being started by a preceding deglutition. The bolus had to be of a certain size; if too small it was either without effect or the effect set in late and the movement was slow and irregular. When the bolus was kept by force in one place for a long time that place lost the promptness of its irritability. It recovered this again, however, a few minutes after the removal of the bolus. I shall not enter upon further particulars except to mention the observation made by Dr. Auer and myself that section of one vagus will remove the secondary peristalsis, while the primary peristalsis is but very little affected.

The chief object of the present communication was a demonstration of both forms of peristalsis in a dog with a permanent fistula in the upper half of the cervical esophagus. I introduced into the fistula an olive-shaped body of hard rubber to which a long thread was attached. The thread ran over a rod and had a paper fan at the opposite end. When the olive-shaped body traveled down into the stomach the fan was observed to move upwards. When the olive-shaped body was placed into the lower half of the cervical esophagus it remained in that place without moving downwards. A deglutition, on the other hand, carried it down into the stomach. But when the olive-shaped body was placed into any part of the thoracic esophagus it was promptly carried down into the stomach without the aid of a preceding deglutition. When the olive-shaped body was held back by force for some time, it was not carried down spontaneously — a deglutition, how-

ever, carried it down promptly. These facts mean that the thoracic esophagus, which remained normally innervated, manifested secondary and primary peristalsis. Retention of the olive-shaped body in one place for some time fatigued the sensory nerve fibers and thus impaired the mechanism of the secondary peristalsis, but the primary peristalsis which required only intact motor nerves remained unaffected. In the cervical part, however, the innervation of the left side of the esophagus was greatly impaired or perhaps even abolished by the operation and the abnormal adhesions.

We see from the last mentioned results, therefore, that the secondary peristalsis is completely abolished, while the primary peristalsis is practically intact, which is in harmony with the above mentioned observations of Dr. Auer and myself of the effect of section of one vagus upon the secondary peristalsis of the esophagus.

### 30 (173)

#### **Peristaltic movements of the rabbit's cecum and their inhibition, with demonstration.**

**By S. J. MELTZER and JOHN AUER.**

*[From the Rockefeller Institute for Medical Research.]*

The rabbit's cecum fills nearly one half of the abdominal cavity and is full of food, which has to get into it and leave it again by some moving force. Nevertheless we find in the literature practically no statement on the movements of that organ. There is good reason for it. When the abdominal cavity of a rabbit is opened the cecum as a rule shows no motion. We wish to report that according to our observations, that organ exhibits well marked and quite regular peristaltic movements; but these can be seen only in the normal animal. When a well fed rabbit is fastened on its back on a holder and the hair of the abdomen is removed, as a rule movements of the cecum can be seen sooner or later. The movements are well marked and characteristic in their appearance, and leave no doubt as to the organ in which they take place. We shall mention only a few details in this communication. As a rule, especially in well fed rabbits, the movements begin in the colon and travel towards the small gut, that is, they are antiperistaltic in

character. But frequently at the end of an antiperistalsis, after only a short interval, the wave returns and runs from the small gut towards the colon; in other words, the antiperistalsis is often followed by a peristaltic wave. The constriction is preceded by a bulging which is more marked than the former. The degree of the constriction (and bulging) is variable. Weaker waves sometimes do not finish the course. A complete course of a wave in one direction lasts from thirty to fifty seconds. The average rate of the movements is about one per minute, but the rhythm is far from being regular.

Some influences suppress cecal peristalsis. Ether applied through the nose stops the movements but they return in about a minute after the ether is removed. Pain, struggle and fright stop the movements; but they soon return again. The most striking effect, however, is the one caused by opening the abdomen: the peristaltic movements as a rule disappear completely and permanently.

What is the cause of this complete abolition of the movements? We thought it might be due to the strong and perhaps continued pain which the laparotomy causes, and tested this theory in the following way. The dorsal cord of a rabbit which showed well defined peristalsis of the cecum was cut at about the third vertebra. As a rule, in such experiments, the peristalsis was stopped for an hour and longer. After the peristalsis had been completely reestablished the abdomen was opened. The laparotomy could now cause no pain; nevertheless it completely abolished the peristalsis, as in a normal animal.

In the course of the latter series of experiments we made the observation that it was not necessary to open the peritoneal cavity to inhibit the movements. Cutting through the skin in the linea alba (in an animal with a cut cord) and dissecting it extensively from the muscles below was sufficient to abolish all cecal peristalsis. Furthermore, the movements returned as soon as the muscles were again covered by the skin, the cut edges of which were held together by clamps. It looked as if the cooling and drying due to the impact of the air upon the muscles above the cecum might have caused the suppression of the movements. But suspending the skin flaps and filling up the cavity above the muscles with warm physiological salt solution did not restore the cecal peristalsis. Furthermore extensive dissection of the skin of the lower



extremities also suppressed these movements. Finally immersion of the lower half of the animal in a warm saline bath inhibited the movements for twenty minutes and longer. When the peristalsis was reestablished it could then again be inhibited by taking the animal from the bath. All the various conditions referred to could affect the cecum only reflexly and not directly.

These experiments led to the inevitable conclusion that the warm or cool bath, and the dissections of the skin over the abdomen and the lower extremities, were various forms of more or less effective stimuli which caused reflex inhibition of the cecal movements. The path of these reflexes could run only through the dorsal cord below the cut. This conclusion was then tested by the effect which the complete destruction of that part of the cord would have upon the inhibitory reflexes. Cecal peristalsis is frequently abolished by such an operation, but reappears sooner or later, and then is often more marked than before the destruction. It was found that after the destruction of the cord the peristalsis of the cecum could not be inhibited by baths, dissections, etc. It was thus established that the cecum is under the control of inhibitory influences invested in the cord, which can be called into action by various peripheral stimulations. Such a stimulus is also exposure to the air of a part of the body which is usually more or less covered.

Under these circumstances we had reason to assume that the inhibitory influence of a laparotomy might be due also to such a stimulation and that it is in the nature of a reflex inhibition. But after further experimenting we found that opening of the abdomen, whether within a saline bath or not, unlike the other peripheral stimulations, inhibits greatly the cecal peristalsis: even after the destruction of the cord, only a few incomplete cecal waves appear after a laparotomy. We must then conclude that direct stimulation of the cecum caused by its exposure to abnormal conditions is capable of inhibiting its movements also directly. Laparotomy therefore abolishes the movements of the cecum by direct inhibition assisted probably also by reflex inhibition.

As to the cause of the movements of the cecum we found that the peristalsis ceased after cutting both vagi. Furthermore stimulation of the peripheral end of one vagus causes a tetanic contraction of the entire cecum, especially after destruction of the cord.

The latter effect is quite peculiar, however. The tetanus lasts only a short time, no matter how long or brief the stimulation may be. Moreover, the effect cannot be obtained by a second stimulation unless quite a long interval passes between the stimuli.

(Some of the above mentioned facts were demonstrated on an animal with destroyed cord.)

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#### **Deglutition through an esophagus partly deprived of its muscularis, with demonstration.**

By **S. J. MELTZER.**

*[From the Rockefeller Institute for Medical Research.]*

As a result of the experiments which Kronecker and I carried out about twenty-seven years ago, it appeared to be conclusively established that liquids are squirted down into the esophagus by the force of the contractions of the mylohyoid muscles and some muscles of the tongue, and that liquid thus projected reaches the cardia long before the arrival of the peristaltic wave. At that time the experiments were carried out on a human esophagus. About ten years ago in a series of experiments on the dog I found that our contention held good also for that animal. Cannon and Moser, however, who studied the esophagus by the fluoroscopic method, although confirming our conclusions for the human being, state that "in the dog and cat but little variation was seen in the swallowing of liquids and solids." Recently Schreiber stated that even in the human being, liquids, just like solids, are not squirted down but are carried by the muscles of the mouth and tongue to the pharynx, whence they are conveyed further into the esophagus by the contractions of the constrictors of the pharynx and are finally transported into the stomach by the peristaltic movements of the esophagus. In other words, liquids are also slowly pushed forward through every section of the path of deglutition by the contraction of the muscle fibers of that section; there is no part of that long path through which liquids are thrown or squirted.

I do not intend to enter into an analysis of the experiments and arguments upon which Schreiber founded his views. The object of

my communication was to demonstrate *a dog drinking in a perfectly normal manner, although a large section of its path of deglutition was deprived of all muscle fibers*. In a number of dogs I have completely removed the muscularis from the entire cervical esophagus. Already on the next day after the operation they drank milk and water like normal dogs. In these cases there were no muscle fibers for quite a long distance to do the slow work of pushing the liquids into the thoracic esophagus. They were apparently squirted through the cervical esophagus by a muscular force located anteriorly to the esophagus. That this force is not due to the constrictors of the pharynx was demonstrated by another experiment. In one dog, besides the removal of the esophageal muscularis, the middle and lower constrictors of the pharynx were cut and completely put out of function. This dog, also, drank without any difficulty the day after the operation. The throwing force is apparently exercised by the muscles of the mouth and tongue.

I wish to call attention to another point. Recently again it was claimed that liquids go down the esophagus by the force of gravity. No experiments were offered in proof of that contention but it had the support of the authority of Von Mickulicz. In my demonstration the bowl of milk was placed on the floor and the large dogs that had been operated on drank from it against gravity without any difficulty.

I would call attention to another matter which has been overlooked by some writers. We have established the fact, and it is easily demonstrated, that each act of swallowing inhibits the peristalsis relating to the preceding deglutition, and when swallows follow one another at intervals of one second there is no peristalsis in the esophagus until after the last swallow. Dogs drink very rapidly, and can take 200 c.c. and more without stopping. Where then is the peristalsis even in normal dogs to carry down such a large quantity of liquid? Does the latter simply accumulate in the pharynx and the upper part of the esophagus until the last swallow?

Finally I wish to say that the essential part of our problem is the *establishment of the theory as it was originated by Kronecker, viz., that besides the slow transportation of food by peristalsis, the function of deglutition is provided with a mechanism for a rapid squirting down of appropriate materials*. As to which of the mechanisms

comes into play in any specific case depends upon the nature of the material which is swallowed. We said that *liquid* is squirted down, but I am quite sure that thick syrup is not squirted farther than the upper part of the esophagus, if so far. We said that semi-liquids or semi-solids are also thrown down. We came to this conclusion from observations made on the swallowing of bread thoroughly softened in water. Possibly in this case a separation took place and the water was thrown down while the bread or some of it stuck to the wall of the gullet and was later gathered up by the peristalsis. It is not improbable that this is what occurs when a mixture of bismuth and water is swallowed. The water may be squirted down, while a large part of the bismuth may stick to the wall and be gathered up later by the succeeding peristalsis — and it is the latter which is probably seen through the fluoroscope.

## CONTRIBUTIONS TO THE BIOLOGY OF DIPLOCOCCUS INTRACELLULARIS.

By SIMON FLEXNER, M.D.

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### INTRODUCTION.

Cerebro-spinal meningitis prevailed as an epidemic in Greater New York City during the winter of 1904 and the spring of 1905. The epidemic embraced about 4000 cases of the disease. The deaths numbered 3,429. In the year 1904, 1,403 deaths occurred from meningitis. In the year 1905, the number of cases of the disease reported was 2,755, and the number of deaths, 2,026. The percentage mortality, deduced from the figures for 1905, is 73.5 per cent.

In the spring of 1905, the Health Department of the City of New York appointed a commission to co-operate with the department in investigating the epidemic. As a member of the commission, I undertook an experimental investigation of epidemic meningitis, out of which the following reports have grown. The rather one-sided nature of the studies, which are to be given here, is to be explained, in part, by the allotment of different aspects of the investigation to the several members of the laboratory committee of the commission.<sup>1</sup>

The particular subject of investigation allotted to me was the pathogenic properties of *Diplococcus intracellularis*, and the immunization of animals with a view to obtaining immune sera which might be used for the differentiation of the diplococcus by the agglutination test. In the course of the study of its pathogenic effects, I became impressed with certain biological properties of the *Diplococcus intracellularis* which have not hitherto been recorded.

In a small number of instances only did I isolate the diplococcus

<sup>1</sup> The laboratory committee of the Commission consisted of Drs. W. J. Elser, E. K. Dunham, J. M. Van Cott, Jr., and Simon Flexner.

directly from spinal fluids obtained by lumbar puncture, or from exudates secured at autopsy. I relied for the greater part of the cultures with which I worked upon Dr. E. K. Dunham, Dr. Martha Wollstein, of the Babies Hospital, and Dr. E. P. Bernstein, of Mt. Sinai Hospital. I frequently obtained from Drs. Wollstein and Bernstein cultures in the first and second generation on serum-glucose-agar. Dr. Dunham provided me with many strains of *Diplococcus intracellularis* and a number of diplococci whose properties were not identical with this diplococcus. These latter were obtained, for the most part, from the nose and pharynx. I secured a number of strains of diplococci, which I compared with *Diplococcus intracellularis*, from the nose and throat of healthy persons, and from domestic pets, cats and dogs, owned by persons in whose homes cases of meningitis occurred.<sup>2</sup>

The difficulties of cultivating *Diplococcus intracellularis* were greatly reduced by the employment of sheep serum instead of human serum. For this advantage I am indebted to a suggestion of Dr. W. H. Park, of the Department of Health. Sheep-serum-water, prepared according to Hiss' method, is mixed with a beef infusion agar-agar containing 2 per cent. of glucose. The quantity of sheep-serum water need not exceed  $\frac{1}{20}$  to  $\frac{1}{10}$  of the volume of the agar. It is added to the sterile and melted agar-agar which is afterwards slanted in test tubes, or allowed to congeal on the expanded surface of 16 ounce Blake bottles. The latter are used for obtaining mass cultures.

The identification of the cultures of *Diplococcus intracellularis*, which I studied, offered no special difficulties. Their origin from

<sup>2</sup>The nasal cavities of the domestic pets were examined by my assistant, Mr. Ward. As a rule, it was necessary to etherize the animals in order to obtain satisfactory "swabs" from the upper nasal mucosa. Several cocci or diplococci decolorizing by Gram's method were obtained but they were readily distinguished from *Diplococcus intracellularis*. Through the kindness of Dr. A. C. Abbott, of Philadelphia, Mr. Ward was enabled to make plate cultures from the upper nasal mucous membrane of fifty inmates of the Philadelphia Hospital. At the time of this investigation meningitis was not epidemic in Philadelphia, and it did not become so afterwards. The inoculated plates were divided with Dr. E. K. Dunham. Neither Dr. Dunham nor Mr. Ward obtained from them any diplococcus which could be confounded with the *intracellularis*. Cocci agreeing with *M. catarrhalis* were, however, obtained from several plates.

the spinal canal of persons suffering from epidemic meningitis was always presumptive evidence of their nature. The diplococci fulfilled the ordinary cultural requirements, and they showed the low degree of viability which has been observed commonly. I quickly learned to lay some stress upon rapidity with which involution occurred in cultures which grew luxuriantly, and, later, I learned to trust the fermentative reactions upon sugars of the diplococcus. Dextrose and maltose are feebly attacked by the diplococcus, and acid is produced. Gas is never liberated, and in a sheep-serum-water-litmus medium, containing the sugars mentioned, reddening, but no coagulation, is produced.<sup>3</sup> Certain other diplococci, not staining by Gram's method, with which *Diplococcus intracellularis* might possibly be confused, were either devoid of power to ferment sugars, or they possessed wider or more vigorous fermentative power than the intracellularis.<sup>4</sup>

#### AUTOLYSIS OF THE DIPLOCOCCUS.

The brief vitality of many cultures of the intracellularis is a point of differential importance. Many strains, grown on a favorable medium, unless transplanted to a fresh medium, do not survive beyond two or three days. Indeed, these transplantations do not succeed, frequently, unless considerable quantities of the growth are carried to the fresh tubes, an indication that only a fraction of the cocci have survived, or are still capable of multiplication. Vigorous fresh cultures of 16 to 24 hours' growth stain sharply and uniformly. On the second day, the cocci show irregularities in staining and size, which irregularities are increased by dessication of the surface of the medium and by other injurious causes. Cultures three days old show marked degenerations, and these increase rapidly with age until, at the end of five or six days, or even earlier, no normal cocci persist. Even with very careful preservation, the great majority of the cocci do not survive beyond this period. A few cocci, in carefully preserved cultures of certain strains, may survive for many

<sup>3</sup>The sugar reactions of the diplococcus have been studied especially by Dr. Dunham and Dr. Elser. (E. K. Dunham, *Jour. Infec. Diseases*, 1906, Supplement 2, p. 10; W. J. Elser, *Jour. Med. Research*, 1906, ix, 89.)

<sup>4</sup>The value and limitation of the agglutination reaction are discussed by Dunham in his report, *Op. cit.*

months. The appearance of old cultures is characteristic. At the beginning of the degeneration, swollen, deeply-stained cocci occur among the smaller but usual forms, which take the stain less intensely than fresh cultures. At this stage, tetradic groups appear, especially among the swollen cocci. As degeneration progresses, loss of staining and disintegration ensue, until, finally, staining power is entirely lost and a formless detritus remains.

The slight viability of *Diplococcus intracellularis* when grown outside the body, and the characteristic changes associated with its dissolution, led me to try the effect of a number of experimental conditions upon these phenomena. The low degree of viability was conceivably due to rapid exhaustion of the medium of growth, at least of that superficial layer of the solid medium upon which growth takes place, or of accumulation of growth products, or of these two factors acting together. The possibility must be kept in mind that the life-history of the diplococcus, within and without the body, may be, at best, a brief one. In this case vigorous-looking cocci would appear in cultures only so long as multiplication was proceeding; and once the cocci have reached the expiration of their brief life-period, disintegrative or involutional changes would set in quickly. Some notion of the influence of exhaustion of the medium and accumulation of metabolic and disintegration products upon multiplication and involution, could be obtained by experiment.

A series of tubes containing the glucose-sheep-serum agar was inoculated with the diplococcus by smearing the slanted surfaces with a recent culture. After twenty-four hours' growth at 37° C., the surfaces were washed with successive quantities of sterile salt solution. Some of the tubes were now returned to the thermostat, and others were returned after the surfaces had been covered with a layer of sheep-serum water and then drained free of the excess. Previous tests had shown me that the diplococcus does not grow in sheep-serum water to which glucose has not been added. Examination of the cultures twenty-four to forty-eight hours later showed that growth had taken place in all the tubes, was less abundant than in the first instance, and presented a filmy, granular appearance. Microscopically, typical diplococci were present; at the expiration of forty-eight hours in the thermostat, the number of involution-forms was large.

Owing to the ease and rapidity with which the diplococcus can be made to undergo dissolution (see below), its soluble disintegration products are readily obtained. These products were tested for their effect upon the growth and vitality of the diplococci.



The large flat surface of medium in a one-pint Blake bottle was inoculated with a culture (654) of *Diplococcus intracellularis*. An abundant growth was obtained in twenty-four hours. About 15 c.c. of sterile 0.85 per cent. salt-solution were poured over the surface, and a few drops of toluol added. The bottle was returned to the thermostat until the next day, at which time an almost clear fluid, devoid of toluol, remained overlying the agar surface from which the growth had disappeared. Cover-glass preparations showed disintegration of all the cocci. The fluid was centrifugalized until clear, and distributed to melted glucose agar as follows: each tube contained 8 c.c. of the melted agar (cooled to 60° C.) to which were added 0.5 c.c., 0.8 c.c., 1.0 c.c., 1.5 c.c. and 2.0 c.c., respectively, of the "extract" of the cocci. The tubes were allowed to cool in the slanting position and each was inoculated with a vigorous culture (656) of the diplococcus. The tabulation shows the result. First observation after twenty-four hours.

Quantity of Extract.	Visible Growth.	Microscopical Examination.	Subsequent History.
0.5 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
0.8 c.c.	None.		Re-inoculated with fresh culture.
1.0 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
1.5 c.c.	None.		Re-inoculated.
2.0 c.c.	None.		Re-inoculated.

The second observation was made at the expiration of a second twenty-four hours. The spread colonies now yielded an abundant growth; the re-inoculated tubes all gave growths in separate colonies which, on microscopical examination, showed vigorous cocci except in case of the tube receiving 2 c.c. of the "extract" in which the dozen colonies of diplococci presented a swollen and involutinal appearance. At the lapse of another twenty-four hours, all the cultures had begun to degenerate or showed more advanced degeneration, the latter being most marked in the tube with 2 c.c. of the "extract." Transplantations to fresh serum-agar surfaces gave growths in all instances.

From these observations, it would appear as if the brief vitality of the cultures grown outside the body is not to be ascribed directly to the exhaustion of the medium of growth, or the accumulation of injurious products of growth, in the short period of twenty-four to forty-eight hours. It may be safely stated that the quantity of disintegration products added to the tubes in the form of "extract" greatly exceeded the possible accumulation under ordinary conditions of cultivation. The higher concentrations of the "extract" undoubtedly inhibited the growth, and exerted an harmful influ-

ence on the morphological appearances of the diplococci. But as the cultures could still be transplanted successfully at the expiration of forty-eight to seventy-two hours, at which period the experiment was terminated, they may be taken to have retained their vitality quite unimpaired.

It has been observed repeatedly that cultures of *Diplococcus intracellularis* tend to survive longer when kept constantly at the thermostat temperature than when kept at varying room temperatures, or in the ice box. That cultures tend to die quickly when placed in the ice box was noted accidentally by me early in this investigation. A series of tests was carried on in order to determine whether any differences could be noted depending, first, upon the concentration of the suspension of cocci kept on ice, and next, upon intermittent variations in temperature between 37° C. and 2° C., as compared with a constant thermostat and refrigerator temperature.

Two heavy suspensions in salt solution of diplococci (596 and 598) were kept in the refrigerator, at a temperature of about 2° C., for five days, at the expiration of which time vigorous and abundant growths were still obtained on transplantation. This result has an interesting bearing on the influence of concentration of the suspension of the cocci on their disintegration at higher temperatures.

Three tubes containing vigorous twenty-hour-old cultures of diplococcus (656) were treated as follows: (a) was kept constantly at 37° C., (b) was alternated daily between the thermostat and refrigerator (2° C.), and (c) was kept constantly in the refrigerator. Experiment began November 8. First transplantation November 10. (a) and (b) abundant growths; (c) moderate growth. Second transplantation November 12. (a) and (b) good growths; (c) small number of separate colonies. Third transplantation November 13. (a) and (b) good growths; (c) no growth.

From these tests certain conclusions can be drawn. In the course of growth of the diplococcus, the medium (if solid) is not exhausted of all nutritive value for the coccus in twenty-four to forty-eight hours; and the products of disintegration of the diplococcus are inhibitive to further multiplication of the coccus only when they have accumulated in large quantities. It would indeed appear as if such an accumulation of growth products as to prevent further development could probably not occur in the course of ordinary vegetative existence of the organism; and it is safe to infer that the short vitality of the coccus in cultures is not due directly to the injurious action of these substances. The tests fur-

ther bring out the fact that a fresh and vigorous culture upon a slanting solid surface succumbs quickly when kept constantly at a temperature just above zero, and is less injured by the wide variation of temperatures between  $2^{\circ}$  C. and  $37^{\circ}$  C. It could, therefore, be shown that cold is inherently injurious to the cocci, and the injury is inflicted not at once that the low temperature is reached, or equally upon the cocci exposed to it. This fact has an important bearing upon the enzymotic activities of the diplococcus to be described presently. In view of the ease and certainty with which solid cultures are destroyed by cold, the greater resistance displayed by highly concentrated suspensions of the cocci in salt solution is noteworthy. Salt solution, even when changed with washings from the surface of a solid medium, probably does not permit any multiplication of the diplococci; besides which any increase is excluded, in this experiment, by the low temperature. That so many of the cocci survived in the strong suspensions may be due merely to greater vitality of a small number of the cocci, or it may, on the other hand, be due directly to the concentration of the fluid which conceivably exercises a protective influence. This factor of concentration of suspension of cocci is very important in respect to the preservation of the cocci under the conditions of higher temperature when enzymotic action may be presumed to come actively into play, and hence it was desirable to determine whether it played a really important part at the lower temperatures.

A milky suspension of a culture "Smith," twenty hours old, on sheep-serum agar was made in salt solution. The suspension was divided in three parts and used for an experiment at  $37^{\circ}$  C.,  $2^{\circ}$  C. and  $-5^{\circ}$  C.

The first experiment to be related was made at  $2^{\circ}$  C. Three tubes were charged with the suspension: (1) was the original strength; (2) contained 0.5 c.c. of the original suspension and 1.0 c.c. salt solution, and (3) contained 0.1 c.c. of the original suspension and 1.0 c.c. salt solution. The first observation was made at the end of twenty-four hours. Cover-glass preparations showed the cocci to be well-preserved in (1), somewhat less sharply-staining in (2) and (3). At the expiration of the second twenty-four hour period, at which time cultures were made, cover-glass preparations showed in all the tubes a moderate reduction in sharpness of outlines and staining of the cocci. The results of the cultivation are important. (1) showed a solid growth of diplococci covering the midportion of the tube; (2) gave sixteen characteristic colonies; (3) gave no growth whatever. Approximately the same volume of suspension, but not the same number of diplococci, was transplanted in each tube.

The deduction from the previous test and this experiment is obvious: cold is injurious to the cocci and destroys cocci in a weak more quickly than in a more highly concentrated suspension. It will not be profitable to discuss at this juncture whether the survival in the concentrated suspension is due directly to its numerical superiority in cocci or indirectly to the existence of more resistant cocci which, as the whole number is more numerous, are also more numerously represented. In even the weakest suspension (3), the cocci transplanted must have been innumerable.

Still further depression of the temperature appears to exert even a greater injury to the cocci. Suspensions of the cocci which were placed, at the same time, at  $-5^{\circ}\text{C}$ . failed entirely to grow when transplanted, forty-eight hours later, to a favorable medium, although the morphological changes in the cocci were relatively slight.

It was stated that salt solution is not a medium of growth and multiplication of the cocci. A series of tests was made with salt solution and other media in order to determine the fate of the diplococci introduced into them. The details of the experiment need not, perhaps, be given. A strain of the diplococcus, which by reason of long cultivation outside the body was able to grow feebly on plain agar, was employed. Salt solution (0.85 per cent.), distilled water, and sheep-serum water were inoculated with small quantities of the culture and examined twenty hours later. The salt solution, and distilled water showed diplococci in a good state of preservation; the sheep-serum water showed no diplococci. At the expiration of the next twenty-four hours, those in the distilled water were in part fragmented and decolorized, those in the other media remaining unchanged. Growths could not be recovered from any of the fluids.

The observation of the persistence of the diplococci in a dead state in salt solution and water for about forty-eight hours without suffering dissolution is interesting when compared with what takes place, under similar conditions, when the suspensions are heavier. I had observed, in the course of experiments on the virulence of the diplococcus that a concentrated salt suspension tended to go off in virulence for guinea-pigs in a few hours, and, as compared with

the state of preservation of solid cultures of the same age, they were more advanced in involution. This observation led me to a study of the influence of concentrations of salt suspension upon the vitality and destruction of the diplococcus, which gave some interesting results.

If a fresh culture, on a favorable medium, is suspended in salt solution to a milky mixture, and the latter is kept at 37° C., the greater number of the cocci introduced will be so degenerated at the end of twelve to fourteen hours as to be almost unrecognizable. As the suspension is diluted progressively, the degree of degeneration and disintegration diminishes. The relative number of diplococci which survive in these suspensions can be determined by cultures at any period. A single experiment will be given here.

A milky suspension of culture (610) was made in salt solution. Microscopical examination showed innumerable diplococci of characteristic appearance. A series of test tubes were charged as follows and placed in 37° C.

(1) Original suspension.	Micro. exam. after 24 hours; disintegration, shadows, fragments.	After 48 hours: no coccal forms preserved.
(2) Original suspension 1.0 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration slightly less than (1).	After 48 hours: some shadowy coccal forms.
(3) Original suspension 0.5 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration less than (2).	After 48 hours: shadows and deep staining cocci remain.
(4) Original suspension 0.1 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: many shadows, no fragmentation.	After 48 hours: best preservation of series.

Cultures made on sheep-serum agar from the suspensions kept at 37° C. for twenty hours gave: Original suspension = no growth; dilution (2) = twelve separate diplococcus colonies; dilution (3) = large number of separate and confluent colonies; dilution (4) = two hundred separate colonies.

If this experiment, which was carried out at the thermostat temperature, be compared with the one similar to it in all respects, except that the suspensions were kept two degrees above the freezing point, it will be seen at once that, however the salt solutions may affect the viability of the diplococcus, the influence of concentration on the viability, is diametrically different under the two sets of conditions given. In other words, at the lower temperature more diplococci survive in the concentrated than in the weaker suspen-

sions, while at the higher temperature, more of them survive in the weaker than in the higher concentration. A glance at the microscopical preparation gives a clew to this difference of behavior: while at the refrigerator temperature the diplococci are not markedly disintegrated, at the thermostat temperature degree of disintegration and concentration go hand in hand. Hence, while the diplococci may lose capacity to grow without losing, at the same time, their form and staining reactions, it is probable that once these latter are lost, viability is also lost. Cold, like salt solution, is inherently hurtful to *Diplococcus intracellularis*; but, besides the injury which the salt solution alone inflicts, another detrimental factor comes into action at the thermostat temperature.

Before considering this factor, the question of the manner in which the salt solution acts may be taken up. It must be borne in mind that as compared with the salt strength of the medium on which the diplococcus is cultivated, the 0.85 per cent. salt solution is hypertonic. A. Fischer<sup>5</sup> has shown that passage from weak into more concentrated salt solution produced in bacteria sometimes plasmolysis, sometimes plasmoptysis, and sometimes neither of these changes. Does, therefore, the change of medium in this case bring out plasmoptytic alterations of the diplococci? Three facts speak against the changes described as being plasmoptytic. The first is the influence of concentration of the bacterial emulsion, the second is the effect of low as compared with higher temperatures, and the third, now to be described, is the influence of a salt solution of 0.9 per cent. concentration containing calcium and potassium chloride. If suspensions of the diplococcus are made in Ringer's solution the viability of the coccus is greatly prolonged as compared with the survival in the salt solution, or, what is even more remarkable, upon the sheep-serum agar. A series of Ringer's solution's suspensions of the diplococcus of three concentrations: (1) Milky suspension, (2) first suspension 0.5 c.c. and Ringer's solution 1.0 c.c., (3) first suspension 0.1 c.c. and Ringer's solution 1.0 c.c., kept at 37° C. survive many days. I have observed that on the twelfth day a considerable number of the diplococci in the original milky suspension was still viable, a smaller number was still viable

<sup>5</sup> *Zeitschr. f. Hygiene u. Infektionskrankh.*, 1900, xxxv, 1.

in the suspension of medium concentration, while no growth was obtained after the fifth day from the lowest concentration. All the diplococci had died out in the original suspension on the fifteenth day. This experiment establishes several important points: it proves that the death of the diplococcus is not caused by hypertonicity of medium alone, by starvation alone, by degree of concentration alone, or by a naturally brief period of vitality. If frequent microscopical examinations of the suspensions in Ringer's fluid are made a gradual dissolution of the diplococci can be made out, but the disintegration progresses slowly, as compared with the effect of salt solution, and the sharp distinction between the different concentrations is lost. I have noticed in those suspensions which gave subcultures a change in appearance of a number of the diplococci which may be connected with the power of survival. These individuals, which are somewhat larger than the average, and take on a deep safranin stain, displaying a high degree of refraction, exhibit a greater resistance to the disintegrative influences. I had already observed these morphological forms in my experiments upon the destructive action of exudates upon the diplococcus, and the question occurred to me whether they represent a more enduring type of the micro-organism. I am unable, at present, to answer this question.

A simple experiment has shown that the addition of a salt of calcium to an otherwise suitable culture medium will greatly increase the period of viability of the diplococcus which has grown upon it. If calcium carbonate is added to the sheep-serum water agar, a given culture will survive many weeks. A rough experiment showed that at the end of five weeks a strain which otherwise survives, on the medium minus the calcium salt, only two, three or four days, was still alive and able to yield abundant sub-cultures. While at the end of five or six days, the original cultures show no preserved, but only disintegrated diplococci; the growth on this new medium two weeks old still showed innumerable diplococci of faint staining power.

The experiment at 37° C. with the Ringer's solution indicated that the survival of diplococci was not necessarily in inverse ratio to the concentration of the suspension, but was in ratio to the con-

centration. In this respect the influence of Ringer's fluid is widely different from that of salt solution, and agrees with the effect of cold on the survival of the diplococcus in salt suspensions. It was deemed desirable to ascertain whether cold, as such, exercised as injurious an influence upon the diplococcus suspended in Ringer's solution as it was shown to exercise on agar cultures and on salt suspensions. Diplococci suspended in Ringer's fluid and kept at 2° C. survived eight to ten days, which is about three times as long as they survived under the other conditions. This period is behind that of survival at 37° C., whence it follows that cold as such is more injurious than warmth. Diplococci could be subcultured longer from the stronger than from the weaker suspensions kept at 2° C.; and as the power to grow in subcultures was lost disintegration of the diplococci was observed to take place extensively. Exceptions to this rule may be observed. A highly concentrated suspension in Ringer's fluid of a vigorous looking diplococcus culture failed, in one instance, to survive 24 hours at 2° C.

It must, therefore, be concluded that salt solution is directly injurious to *Diplococcus intracellularis*, as it has been shown by Jacques Loeb<sup>6</sup> and others to be poisonous to many animal cells; and it must be believed that the toxic effects of sodium chloride can be neutralized by certain calcium and potassium salts. Although the salt solution causes directly the death of the diplococci, it would appear that it is not to it, but to some other agency, to which must be ascribed the disintegration which rapidly overtaxes the dead micro-organisms.

The nature of this agency is rendered fairly evident from the microscopical appearances presented by the suspensions: it would seem to be enzymotic. The manner of dissolution of the diplococci suggests this, and the enzyme is doubtless contained in the bacterial cells. As enzymes act with energy not upon highly vitalized, but upon dead or partly devitalized cells, the question arises whether in the case of the diplococcus the enzyme can act immediately as a devitalizing as it can as a dissolving agency. It has been shown that in weak salt suspensions the diplococci survive and retain their morphological integrity longer than in stronger suspensions; and

<sup>6</sup> *Biochemische Zeitschrift*, 1906, ii, 81.



hence, it may be conceived that while the charges of enzyme which individual diplococci carry may be inadequate both to destroy and to disintegrate them, that in the more concentrated suspensions the enzyme liberated from a larger number of disintegrating micro-organisms, may exert a devitalizing effect. The intracellular enzyme is, therefore, a dissolvent of dead diplococci, and in certain states of concentration a poison for living ones.

The action of the enzyme upon the bacterial cells can be accelerated by the employment of chemical and physical agents which serve to kill the diplococci without, at the same time, seriously injuring the enzyme. Heat, carefully applied, and toluol have been used to accomplish this purpose. Suspensions of the diplococcus heated for thirty minutes to the temperature of 60° C. are killed and still capable of undergoing disintegration or autolysis. The ultimate result is the same whether the suspension has been made in salt or Ringer's solution; while in point of rapidity the dissolution is somewhat slower than in strong unheated suspensions in salt solution. Heating to 65° C. and higher temperatures, reduces the capacity of the diplococcus to undergo rapid disintegration, probably because the enzyme is weakened or destroyed.

If to a strong salt suspension of the diplococcus toluol be added and the mixture kept at 37° C., the morphological changes indicative of autolysis begin to be evident in one hour, to be pronounced in two hours, and nearly complete in four hours. Ringer's fluid has no marked influence on this form of autolysis. It is interesting to note that the degree of concentration of the suspension influences the result in the presence as in the absence of toluol. Even the most concentrated salt suspensions alone do not show appreciable changes in the morphology of the diplococcus at the expiration of four hours at 37° C. The tabulation which follows brings out the main facts.

If Series I and II in the succeeding tabulation are closely scrutinized, such differences in degree of disintegration can be made out as to indicate that toluol not only accelerates autolysis of the cocci, but it tends also to make it more complete and to minimize, but not to set aside entirely, the effect of the degree of concentration upon the final disintegration.

A milky suspension of diplococcus (656), showing under the microscope innumerable diplococci, was prepared. Series I consisted of salt suspensions of cocci alone, Series II of salt suspensions to which toluol was added. The examination was made after eighteen hours at 37° C.

SERIES I.

Original suspension.	Extensive disintegration; small number of coccal forms still visible.
Original suspension, 1.0 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.5 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.2 c.c.; salt sol. 1.0 c.c.	Fair degree of preservation of cocci.
Original suspension, 0.1 c.c.; salt sol. 1.0 c.c.	About same degree of preservation as previous one.

SERIES II.

Original suspension + toluol.	Complete disintegration.
Original suspension 1.0 c.c.; salt sol. 1.0 c.c. + toluol.	Complete disintegration.
Original suspension 0.5 c.c.; salt sol. 1.0 c.c. + toluol.	Disintegration incomplete; pale and irregular coccal forms visible.
Original suspension 0.2 c.c.; salt sol. 1.0 c.c. + toluol.	Somewhat better preservation than previous one.
Original suspension 0.1 c.c.; salt sol. 1.0 c.c. + toluol.	Better preservation than previous one.

Jacques Loeb<sup>7</sup> has drawn attention on several occasions to the restraining influence of potassium cyanide upon intracellular enzymotic activity. The diplococcus offered, therefore, the opportunity to study the effects of this chemical body upon an active autolytic ferment under the conditions of purity of culture of micro-organic cells—conditions probably not obtainable with the cells of higher living forms.

The experiments were made with different strengths of the cyanide and suspensions of the diplococci. Potassium cyanide in 1/200 per cent. solution kills the diplococci outright. If now the diplococci, in watery suspensions, are left in contact with the cyanide, either with or without the addition of toluol, the organisms do not disintegrate, and suffer only reduction in their affinity for

<sup>7</sup> *Biochemische Zeitschrift*, 1906, i, 183.

dyes. That enzyme activity is not wholly suppressed by the cyanide would seem to be indicated by the better state of preservation of the cocci in the dilute suspensions. The tabulation (Series III) brings out this difference. The suspensions and examination of the diplococcus were made at the same time as those of Series I and II of the preceding tabulations.

SERIES III.

KCN 1/20% 1.0 c.c., susp. cocci 1.0 c.c.	Outlines of cocci pale but visible; possibly reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.5 c.c.	Fair preservation of the cocci; no reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.2 c.c.	Good preservation of the cocci.
KCN 1/20% 1.0 c.c., susp. cocci 0.1 c.c.	Very good preservation; outline of cocci not perfectly distinct.
KCN 1/20% 1.0 c.c., susp. cocci 0.01 c.c.	The same as previous one.

This experiment shows that potassium cyanide possesses the power of preventing or diminishing autolysis of the dead diplococci. In the case of the higher concentrations it is even possible that a greater quantity of the cyanide may have wholly suppressed autolysis. Doubtless many other chemical agents possess this power of suppressing autolysis of the diplococcus; and hence it was necessary to ascertain whether the removal of the cyanide will admit the enzyme to reassert its enzymotic action, as, according to Loeb, happens in the case of the eggs of the sea-urchin. If, therefore, the cyanide acts by holding the enzyme in check and not by destroying it, or changing the cell-substance, so as to make it permanently resistant to the action of the enzyme, the diplococci should be restored approximately to their normal condition of autolysis upon the removal of the cyanide.

Ten cubic centimeters of a strong suspension of the diplococcus in salt solution were mixed with one cubic centimeter of a one twentieth per cent. potassium cyanide solution, and the mixture kept at 37° C. for one hour. The cocci and fluid were separated by centrifugalization and the coccal residue washed twice with 10 c.c. of salt solution. The sediment now showed a large number of well-preserved, deeply-staining diplococci. The sediment was resuspended in salt solution and divided into two portions, to one of which toluol was added. They were placed at 37° C. and examined at the end of twenty-four hours and forty-eight hours. No marked difference was noticed in the toluol and non-

toluolized specimen. The diplococci appeared reduced in number, evident disintegration was in progress, and the sharpness of staining had been lost at the first examination. At the second examination, the staining was still more feeble, but no other change was noticeable.

From the experiments, it can be stated that the diplococci, after treatment with the cyanide can be partially restored to their previous state of autolysis. The experiment is not conclusive of the actual degree of restoration possible, since the disintegration of the cocci was incomplete in the experiment. The concentration of the resuspension, which was not high, may explain the imperfect disintegration. It would, indeed, be possible to conduct this experiment quantitatively and ascertain the degree of permanent injury exercised by the cyanide upon the diplococcus; but the object of the experiment in establishing the restraining action of potassium cyanide upon the autolytic enzyme, and the possibility of setting this action aside by mechanical removal of the poison, was attained by the imperfect experiment.

The existence of an autolytic enzyme in *Diplococcus intracellularis* capable of destroying its own cell-substance having now been established, the next step was to ascertain whether the enzymotic action was specific and limited to the diplococcus, or whether it could be exerted upon other bacterial cells. It is known that the diplococcus does not produce a proteolytic enzyme acting upon and dissolving gelatine and coagulated serum. Hence, its enzyme differs materially from the enzymes secreted by certain liquefying bacteria. It seems quite certain that the rapid and striking morphological changes taking place in cultures of the diplococcus are caused, directly or indirectly, by the intracellular enzyme; and it may, therefore, in view of this fact and the related ones already described, be presumed that the diplococcal enzyme is able to break down the complex structures contained in living micro-organisms.

The enzymotic action of the diplococcus was tested upon the two classes of bacteria represented by those staining and those decolorizing by Gram's method. Among the latter, *Bacillus typhosus*, *Bacillus coli communis*, *Bacillus pyocyaneus*, *Micrococcus catarrhalis* and two unidentified Gram negative cocci from the monkey's nose were tested; while among the former *Staphylococcus aureus* and *Bacillus anthracis* were chosen.

The experiments to determine this point were made in the following manner: Fresh growths upon agar-agar surfaces were suspended in salt solution to a milky emulsion which was divided into two equal parts. To one part an equal volume of salt solution, and to the other the same volume of a milky suspension of *Diplococcus intracellularis* was added. Control cover-glass preparations were made immediately and double-stained with gentian-violet and safranin (Gram's method).

Toluol was now added, and the tubes placed at 37° C. under rubber caps. The examinations were made by means of cover glass preparations (Gram's method), usually at twenty-four hour intervals. A few detailed results follow.

Non-Gram coccus from monkey. (a) Controls: Original suspension shows many sharply-staining cocci, single and in pairs; mixed suspensions of coccus and diplococcus permit easy distinction by the morphological differences. After twenty-four hours: Original suspension shows no solution or disintegration of the cocci; outlines of the cocci not quite sharp. Mixed suspension: All the diplococci have disintegrated; the larger cocci from the monkey have lost stainable substance and have run together into small masses in which the individual outlines are indistinct. After forty-eight hours: Original suspension, cocci somewhat paler and cohering slightly. Mixed suspension: Reduction in number of cocci; those remaining appear as shadows. The non-Gram coccus (b) gave results which were almost identical with those of (a).

*B. typhosus* and *B. coli communis* in milky suspensions under toluol, are somewhat altered in appearance at the end of forty-eight hours at 37° C.; while in the presence of an emulsion of the diplococcus, such as was used with the non-Gram cocci from the monkey, they are completely or almost completely destroyed in this period. At the expiration of the first twenty-four hours, the disintegration is advanced.

*Bacillus pyocyaneus*.—The control under toluol showed, at the end of twenty-four hours, no reduction in number, but less deep staining than the preparation made immediately after suspension. The suspension with the diplococcus showed, at this time, reduced numbers, and only shadowy remains of bacilli. At the expiration of the second twenty-four hour period, the control tube still showed many stainable bacilli (number apparently less than in original suspension), while the mixed tube containing the two organisms was completely disintegrated.

Two cultures of *Micrococcus catarrhalis*, which were found to be quite resistant under toluol, were found to disintegrate quickly (in twenty-four hours) under the influence of the suspension of the diplococcus.

*Staphylococcus aureus*.—Control (under toluol) showed no appreciable change after several days at 37° C. The mixed suspension of staphylococcus and diplococcus was examined at twenty-four hour periods for five days. After twenty-four hours, there is reduction in numbers of the staphylococci, although many

still remain. In size these are larger than in the control tube, and the violet staining is less intense. In the succeeding days, the number of cocci further diminished, the cocci coalesced into clumps, and showed still further reduction in power to hold the violet stain. Here and there throughout the preparation, transitions from staphylococci holding the violet stain imperfectly to staphylococci which had taken up the counter-stain (safranin) were observed. At the expiration of the experiment, not all the staphylococci had disappeared or had been decolorized.

Anthrax bacillus twenty hours old on slant agar; no spores visible. Suspended as before: First observation after twenty-four hours. Control (under toluol): Partial and moderate degree of plasmolysis. The threads and felted masses of bacilli show clearly an outer-stained (safranin) cell-wall and an inner-stained (violet) cell-substance. The inner substance is somewhat fragmented and irregularly contoured. Mixed suspension: Advanced plasmolysis and loss, in high degree, of Gram-staining inner substance. The outer cell-membrane is completely gone in many places, and the fragmented or partially dissolved inner substance lies free. Second observation, after forty-eight hours: Control unchanged. Mixed suspension: Practically complete decolorization of the threads, with very few fragmented remains of violet-staining inner cell-substance.\*

It is established by the foregoing series of tests that the enzymotic action of the diplococcus can be exercised on a considerable variety of bacteria, and the observations are not without interest as indicating that micro-organisms, such as *Staphylococcus aureus*, *Bacillus anthracis*, and *Bacillus pyocyaneus*, in spite of their energetic proteolytic functions, show less power to cause disintegration of their own protoplasm than is effected by *Diplococcus intracellularis*. This difference points to a distinction between autolytic and proteolytic bacterial enzymes. The above tests also showed that a great difference exists in regard to the ease and completeness with which this hetero-disintegration of bacterial cells is produced by the diplococcus. Of the bacteria studied which were at all subject to the enzyme *Staphylococcus aureus* offered the greatest resistance; and I think it not improbable that the Gram-positive bacteria in general may prove to be less readily broken down by the diplococcus enzyme than the Gram negative bacteria.<sup>3</sup> Eventually, the bacteria which produce proteolytic enzymes, and, indeed other bacteria, suffer more or less complete disintegration. In the case of *Bacillus*

\*The destruction of *Staphylococcus aureus* was incomplete, the change in *Bacillus anthracis* was one of degree of disintegration; the changes in the other bacteria were more striking. No effect was noticed in tests with *Bacillus tuberculosis* and *Bacillus Moelleri* (timothy bacillus).

*pyocyaneus*, in salt suspension in toluol, the time required for disintegration is, at most, a few days. But the slower forms of involution and degeneration observed in artificial cultures, may or may not be of the nature of the autolytic changes described. These involutional alterations are probably of extremely subtle nature; but even here enzyme activities of quite low grade may be the chief agencies at work.

The heat lability of the enzyme has been studied in two ways. First, the temperature at which the diplococcus is prevented from undergoing characteristic autolysis was determined. Next, the

The suspended contents in sheep-serum water of surface growths from two-pint Blake bottles were distributed among ten test tubes; each tube received about 6 c.c. The tubes were, with the exception of the controls, heated for thirty minutes to temperatures ranging from 55° C. to 90° C. Cultures were made to determine whether any diplococci remained viable. Only the control (unheated) tubes gave growth. Cover-glass preparations were made from the control tubes, and from the heated tubes immediately after heating and at the subsequent periods. The tabulation gives the results:

Suspension.	Immed. Microscop. Appearance.	Microscop. Appearance 24 Hours Later.	Sedimentation After 3 Days.
Control.	Well-preserved cocci.	Marked degeneration; diminution of cocci.	Cloudy; no sediment.
Toluol control	Well-preserved cocci.	A few minute, pale fragments remain.	Almost clear; no sediment.
55°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
60°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
65°C.	Some frag. and decol. cocci.	Somewhat more fragmentation than at 55°C.	Almost transparent; much sediment.
70°C.	Some frag. and decol. cocci.	Forms better preserved than at 65°C.	Almost transparent; much sediment.
75°C.	Some decolorization.	Preservation very good.	Almost transparent; much sediment.
80°C.	Considerable fragmentation.	Preservation good, but much irregularity in staining.	Almost transparent; much sediment.
85°C.	Frag. and apparent diminution in number.	Staining deep; no shadows; irregular forms.	Almost transparent; much sediment.
90°C.	Frag., diminution in number; decolorization.	Forms pale; no further diminution.	Almost transparent; much sediment.

temperature required to render the autolysate of the diplococcus inactive upon other bacterial species was ascertained.

A temperature of 55° C., maintained for thirty minutes, kills the diplococcus, but it does not suffice to remove its capacity for self-digestion. Even after exposure, for the same period, to a temperature of 60° C., the diplococcus undergoes autolysis. This test was several times repeated with different strains of the diplococcus and autolysis was always observed to occur, although somewhat less quickly and perfectly than in unheated coccal suspensions under toluol. Toluol accelerates, I believe, dissolution, by causing plasmolysis and thus promoting the disintegrative effect of the enzyme. The thermal death points of cocci and enzyme are widely separated. Temperatures of 40° C. to 45° C. are capable, if maintained some hours, of killing the diplococci, while the enzyme does not suffer injury under the same conditions. Temperatures of 65° C. and upwards, while causing more or less immediate plasmolysis (or plasmoptysis) prevent further disintegration due to a vigorous action of the enzyme. As the tabulation indicates, two criteria can be used to determine the point of heat-destruction of the enzyme: first, the degree of dissolution shown by the microscope, and next the amount of sediment appearing in the tubes. The two effects stand in a quantitative inverse ratio to each other. The greater the amount of sediment, the smaller the amount of disintegration of the diplococcus as shown by the microscope. Hence the gross test is well adapted to the study of the lability of the enzyme, since it permits of the elimination of any error arising from mere physical or mechanical change in the diplococci.

To ascertain whether the temperature at which self-digestion of the diplococcus is inhibited represents the temperature of heat-destruction of the enzyme, another series of tests was made.

The point of heat injury or destruction of the enzymotic fluid was determined by preparing, in pint Blake bottles, mass cultures of the diplococcus and causing them to autolyze at 37° C. for forty-eight hours in salt solution and in water under toluol. At the expiration of this period the toluol had evaporated. The salt autolysate was distributed in test tubes, of which some were heated to 70°, 80°, 90° and 100° C. for thirty minutes, and some remained unheated. A coagulum formed in the tubes heated to 90° and 100° C. The watery autolysate was not heated. The contents of all the tubes were centrifugalized; and except



those heated to 90° and 100° C., which became transparent, the fluids remained opalescent. Twenty hours' growths of *B. coli communis*, *B. typhosus* and *B. anthracis* were suspended in Ringer's fluid to milky emulsions. The tests were made as follows: A set of test tubes was charged with the unheated salt and water autolysates and the salt autolysate heated as described. To the control tube an equal amount of salt solution was added. An approximately equal amount of the milky bacterial emulsion was now placed into each tube, toluol was added, and the mixtures kept at 37° C. for forty-eight hours. Two examinations at twenty-four-hour periods were made. The result in the case of *B. coli communis* was definite: the control salt suspension showed the bacilli swollen, pale and shadowy, but not disintegrated; the salt and water unheated autolysates showed complete disintegration of the bacilli; the autolysate heated to 70° C. was scarcely to be distinguished in effect from the unheated; while the autolysates heated to 80° C. and higher produced about the same degree of change as the salt control. The result in the case of *Bacillus typhosus* was disappointing, as, in this experiment, the salt control as well as the autolysate mixtures disintegrated completely. A perceptible difference was manifest in the degree in which *Bacillus anthracis* disintegrates in toluolized salt solution and in a mixture of equal parts of salt and Ringer's solution. The addition of the latter reduced the spontaneous disintegration. In like manner the unheated autolysates are less active destructive agents where Ringer's fluid is present, as compared with their action in plain salt solution. And yet the disintegration was greater when the unheated autolysate was present. The autolysate heated to 70° C. and 80° C. produced less change, and that heated to 90° C. and 100° C. still less, than took place in the unheated salt and Ringer's fluid control.

While this experiment is not perfectly sharply cut, it yet indicates that heating the fluid autolysate to 70° C. and above reduces its powers of causing disintegration of certain bacteria.

#### PATHOGENICITY OF THE DIPLOCOCCUS.

*Diplococcus intracellularis* is, as tested on laboratory animals, a micro-organism of low and variable pathogenic action. I have studied its action upon mice, guinea-pigs, rabbits and monkeys. Different strains of the diplococcus were injected into different regions of the body to determine the fatal doses and the pathological effects produced by the micro-organism. In the case of the monkey the inoculations were made directly into the spinal canal, in order to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. The experiments to be related immediately refer to the action of the diplococcus upon the animals mentioned, except the monkeys. The experiments on monkeys form the subject of a separate communication.

A considerable number of strains of *Diplococcus intracellularis* have been tested for virulence upon mice and guinea-pigs. In all the later experiments upon the virulence of the micro-organisms I preferred to employ small guinea-pigs, as being more susceptible to the action of the diplococcus. The difference in resistance to infection in guinea-pigs, depending upon weight, is considerable. Guinea-pigs of 175 to 200 grams weight have proved highly susceptible to the diplococcus, compared to the resistance displayed by pigs weighing 350 to 400 grams. Mice of about 15 grams weight often withstand larger doses of the diplococcus than the smaller pigs.

Not all cultures of the diplococcus, even in the first generations, are even moderately pathogenic for guinea-pigs and mice, although it is always possible to kill these animals by inoculating an excessively large quantity of cultures of low virulence. Speaking generally, the freshly isolated cultures are much more virulent than cultures grown for a period on artificial media. Occasionally, a strain of the diplococcus will retain its virulence for many months, but, as a rule, this power becomes greatly diminished, or is lost, after the first transplantations extending over a few weeks. I have worked with many cultures which deteriorated in a few days, and one culture (Smith) that retained its virulence for many months, to lose it in the end, and another (656) that displayed no diminution in activity after several months cultivation on sheep-serum agar. The "Smith" culture was passed through many mice, guinea-pigs and monkeys during its active period; but once its power was lost, it could not be restored to virulence by passage through mice or guinea-pigs. It still yields, however, by autolysis, an active extract and can be used as an adjuvant to increase activity of other living cultures.

In any comparison of virulence little weight can, I think, be laid upon the absolute quantities of diplococci injected. The animals are, on the whole, so refractory that the number of diplococci that must be injected to produce striking results is, even with the most active cultures, very large. Very little accuracy could in my work be secured through the use of a standard *oese*, for the reason that the contained water in the surface growth was subject to wide

fluctuations, depending upon the strain of diplococcus, composition of the culture-medium, and other external factors.<sup>9</sup> Hence, I substituted for the *oese* a suspension of the diplococci, incubated for a uniform period (usually 20 hours) upon a standard medium (7 c.c. glucose-agar to 1 c.c. sheep-serum water). The suspensions exhibited a degree of opacity which was approximately equal. Select-

No. of Fig.	Weight in Grams.	Denomination and Quantity of Culture.	Result.
113	165	0.1 c.c. No. 654.	Survived.
119	160	0.2 c.c. "	"
121	177	0.5 c.c. "	Died 9 P.M. same evening.
122	192	0.1 c.c. No. 88.	Survived.
127	175	0.2 c.c. "	Died.
128	182	0.5 c.c. "	"
145	197	0.1 c.c. Buck.	Survived.
150	195	0.2 c.c. "	"
159	170	0.5 c.c. "	Died.
161	165	0.1 c.c. No. 9.	Survived.
162	175	0.2 c.c. "	"
163	166	0.5 c.c. "	"
164	161	0.1 c.c. Connolly.	Survived.
166	190	0.2 c.c. "	"
172	195	0.5 c.c. "	Died.

<sup>9</sup> An attempt was made with four cultures of the diplococcus to increase the virulence by growing the diplococci in a glucose-bouillon medium containing one twentieth of its volume of immune goat serum. For the controls normal goat serum was employed. Each culture was passed at twenty-four-hour intervals through ten successive tubes of medium. The tenth cultures were injected into the peritoneal cavity of mice. As the tabulation indicates, no augmentation was accomplished. The immune serum caused agglutination of the growing diplococci. The mice dying in twenty-four hours showed, as is usual, general infection with the diplococcus. Mr. Herbert Ward assisted me in these experiments.

Quantity of Culture Injected.	Immune Series Result.	Normal Series Result.
Kepp culture. { 3 c.c. 2 c.c. 1 c.c. 0.5 c.c. 0.1 c.c.	Died in 15 hours. Survived. " " Died in 5 days.	Died in 10 days. Survived. Died in 10 days. Survived. Died in 10 days.
Behren culture. { 3 c.c. 2 c.c. 1 c.c. 0.5 c.c.	Died in 15 hours. Survived. Died in 24 hours. Survived.	Died in 24 hours. Died in 20 hours. Survived. "

ing a lot of guinea-pigs of about equal weight approximately uniform quantities of the cultures could be injected.

The preceding tabulation will suffice to show the method of testing comparative virulence and give the results obtained in a typical experiment.

One agar-slant culture of each strain was suspended in about 3 c.c. of salt solution. Injections made intraperitoneally at 5 P. M. The pigs succumbed during the night or the next morning.

As salt solution is evidently an active poison for the diplococcus, and as sheep-serum water favors, when added to an otherwise suitable medium, the growth and preservation of the diplococcus, an experiment was made to ascertain whether suspending the diplococci in one or the other fluid influenced the pathogenic result. The experiment was made with a culture on sheep-serum agar eighteen hours old. The suspensions were prepared at 11 A. M., and the injections given intraperitoneally at 4 P. M. In point of concentration the salt suspension was, as indicated by the microscopical field, considerably the heavier.

No. of Pig.	Weight in Grams.	Quantity of Suspension Injected.	Result.
102	230	1.0 c.c. salt.	Died on second day.
121	267	0.5 c.c. "	Survived.
134	260	1.0 c.c. serum.	Died on first day.
130	270	0.5 c.c. "	Died on second day.
190	115	0.2 c.c. "	Died on first day.

This result merely suggests that as compared with a sheep-serum water suspension the salt suspension of diplococci suffers a more rapid deterioration. In certain experiments on immunity which I carried out I observed that it was unsafe to carry the salt suspensions of the diplococci over, from one day to the next, without making a new determination of the strength. The apparently better preservative action of serum-water over salt solution is the more remarkable in that the serum water is not a favorable medium of growth for the diplococcus. Indeed, many strains are unable to develop in it alone; and the transplanted diplococci, which do not multiply, soon disappear. If equally heavy suspensions, say an agar-slant surface growth per 4 c.c. of fluid, of the diplococcus are made in salt solution and sheep-serum water and kept at 37° C.,

the disintegration is more advanced at the end of twenty-four hours in the salt than in the serum-water.<sup>10</sup>

In studying the pathogenic action of the diplococcus salt suspensions were invariably used. Where a large number of animals was to be inoculated, the diplococcus was grown on the flat agar-covered surface of a one-pint Blake bottle. A suspension of the growth in 12 c.c. of fluid was made of which the fatal dose varied, according to the virulence of the diplococcus, from 0.1 to 0.5 cubic centimeter for small guinea-pigs, by intra-peritoneal inoculation. Death from these doses occurred, as a rule, in less than twenty-four hours. Active cultures, in this strength of suspension, may in the smallest dose given cause death in from eight to ten hours. A culture of which a dose of 0.5 c.c. requires twenty-four hours to cause death is one of low virulence. In the course of any large series of experiments, irregularities in the reaction of guinea-pigs to inoculation occur. On the whole, the irregularities occasioned by greater or less resistance on the part of the small pigs are remarkably few. It has, indeed, rarely happened that a guinea-pig of a series died in four hours and another survived thirty-six hours. But these irregularities can, when required, as in tests of immunity, be ruled out by making duplicate inoculations. Care should be exercised not to employ guinea-pigs weighing less than 150 to 160 grams, the most useful weights being about 200 grams. Among the pigs surviving inoculation immediately, it happened, now and then, that death took place after several days, or a few weeks, and was preceded by a state of great emaciation. No constant pathological condition, the emaciation excepted, was found in these animals. The peritoneum was usually clear, and no diplococci remained there or elsewhere in the body.

One of the earliest visible results of the inoculations is a marked reduction in temperature. This is associated, at times, with the characteristic external appearance of sick guinea-pigs: the tendency to avoid the light, and to sit crouched in a corner with the hair erected. The abdominal muscles become tense and hard and the abdomen distended. The irritative effects of the inoculation upon

<sup>10</sup> The directly toxic action of sodium chloride upon the diplococcus has already been described.

the intestine are associated occasionally with prolapsus of the rectum which condition is followed invariably by death. The rapid onset and high degree of reduction of temperature following inoculation permit frequently the prediction in two or three hours of the probable outcome. Hence, it is possible, in many cases, to determine in a short time the result of tests upon virulence of the diplococci, the protective value of immune sera, etc. In testing immune sera it is necessary in each instance to make a preliminary estimation of the activity of the organisms, since the concentration of the suspension and the degree of virulence are such variable factors; and as the suspensions cannot be kept in salt solution for twenty-four hours without changing, and should be injected as soon as possible after preparation, it is an advantage to have this relatively safe guide to further inoculations of the temperature depression. The tabulation will exhibit the effects described.

No. of Pig.	Weight in Grams.	Amounts of Inoc. 12 M.	Temp. Before Inoc.	Temp. and Condition 3 P. M.	Result.
113	170	0.1 c.c. suspension.	38° C.	35.3° C. abdom. tense.	Died 12 M. next day.
119	160	0.2 c.c. suspension.	37.6° C.	34.1° C. " "	Found dead 7 A.M. next day.
121	186	0.3 c.c. suspension.	38.1° C.	35.1° C. " "	Died 9:30 P.M. same day.
122	170	0.5 c.c. suspension.	38.1° C.	34.8° C. " "	Died 9:30 P.M. same day.

As a control for this series the following tabulation will suffice. The temperature of the pigs prior to inoculation was about 38° C. The quantities of emulsion of the several strains of diplococci injected ranged from 0.1 to 0.5 cubic centimeters. The injections

Weight in Grams.	Quantity of Suspension Injected.	Temp. after Inoc.	Result.
165	0.1 c.c.	36° C.	Survived.
160	0.2 c.c.	37.2° C.	"
182	0.5 c.c.	35.6° C.	Died.
{ 165	0.1 c.c.	35.4° C.	Survived.
{ 175	0.2 c.c.	38.6° C.	"
{ 166	0.5 c.c.	38.4° C.	"

were made at 6 P. M., and the temperature of the surviving pigs taken the next morning.

The value of the tabulation immediately preceding consists, first,

in showing that the temperature depression is less in cases in which the inoculation is not fatal, and second, the degree of resistance of the individual pigs is a factor to be reckoned with. The bracketed series illustrates the latter point: the same suspension of diplococcus was injected into the three pigs, and the pig which received the smallest dose showed the greatest depression of temperature. A similar effect has been noted in other cases, but, on the whole, is rather exceptional.

The fate of the diplococci injected into the peritoneal cavity in guinea-pigs depends upon several conditions: the quantity of diplococci injected, the virulence of the diplococci, somewhat upon the individual resistance of the pig, since this is a factor in prolonging or diminishing the period of survival, and the extraneous condition of elapsed time between death and autopsy. As will appear later, the diplococci are not highly resistant to the destructive action of the fluids of the body whether they act upon them in the peritoneal cavity or in the test-tube. Some experiments tend to show that the more virulent diplococci resist the disintegrative action of the fluids in the body longer than the less virulent ones. Comparison of the conditions arising in the peritoneum, depending upon the quantities and the virulence of the diplococci injected, offers a possible explanation of this difference.

The more virulent the diplococcus the shorter is the period of survival after inoculation. Guinea-pigs which succumb in less than eighteen hours—and many succumb to injection of virulent diplococci in ten to fourteen hours—tend to develop a peritoneal exudate poor in leucocytes; while the pigs which survive longer, *i. e.*, from twenty-four to thirty-six hours, tend to exhibit turbid exudates rich in leucocytes. No absolute ratio between the number of leucocytes in the exudate and the degree of disintegration of the diplococci exists; but a relative ratio seems to exist. With an increasing period of survival there is greater emigration of leucocytes and also greater disintegration of diplococci. Hence, the degree of emigration of leucocytes comes to be a measure, in part, of the degree of virulence of the diplococci. The relation can, however, be disturbed either by the inoculation of very large quantities of little virulent, or sublethal doses of the more virulent diplococci, as

might indeed be expected. It is, however, to be pointed out that large overdoses of the weaker diplococci can be readily disintegrated in the peritoneal cavity, even though emigration of leucocytes is largely prevented by the excessive dose. From this fact the result may be deduced that the destruction of the diplococci does not take place necessarily within leucocytes, but can be accomplished by fluid inflammatory exudates alone. As will be seen presently, test-tube experiments bear out this deduction. There is similarity in the pathological conditions met with in cerebro-spinal meningitis in man and in the experimental infection in guinea-pigs. In both species the immediate local reaction to the presence of the diplococcus in the serous cavities is an exudation of fluid, the character and quantity of which varies greatly, perhaps with the quality of the infecting diplococcus. The less cellular fluids often contain the greater number of diplococci of well-preserved appearance which are readily cultivated; while the more cellular fluids often show fewer diplococci and yield cultures with greater difficulty. No striking relation need exist between the virulence of the diplococcus as displayed for man and animals, since the inherent qualities in the germ which determine pathogenicity are often quite dissimilar for different classes of animals.

I have pointed out that some relation between virulence of diplococcus, period of survival of the inoculated guinea-pigs and the emigration of leucocytes exists, and it remains to add that phagocytosis, in the peritoneal cavity, while a variable factor is a fairly constant one. When emigration of leucocytes is at all marked, even with an almost entire preservation of the diplococci, phagocytosis is occurring. When there has been considerable, although not total, disappearance of the free diplococci, phagocytosis is likely to be more abundant. It rarely happens that all the diplococci are within phagocytes unless a small number only of those injected remain. On the other hand, when overdoses of little virulent diplococci are injected they may disappear as stated almost wholly from the peritoneal cavity although the emigration of leucocytes may have been entirely suppressed; or, as is also possible, the emigrated leucocytes may have undergone complete lysis. Hence, I have concluded first, that phagocytosis, in this experimental disease, is a



function of emigration but not a measure of it, and second, the disappearance of the diplococci from the peritoneal cavity does not depend wholly on phagocytosis. I believe, indeed, that the diplococci can be removed, without direct intervention of phagocytes, through self-digestion and digestive action of the inflammatory exudate. The ease with which the diplococcus undergoes autolysis has already been dealt with; and that the inflammatory exudate exerts actively destructive effects upon the diplococcus will be shown presently.

Leucocytes are numerous in every fibrinous exudate, and frequently, in the omentum, when they may be sparse in the peritoneal fluid. Speaking generally, the clear peritoneal fluids contain few leucocytes relatively, and the turbid fluids contain many. The leucocytes of the omentum are large and pale, and those of the peritoneal fluid small and dense. Their nuclei are often irregular in form, or fragmented. Diplococci are common in the protoplasm and rare in the nuclei, and they show all transitions from well-preserved, sharply-staining organisms to fragments of cocci and swollen, metachromatic bodies. Degenerations are exceedingly frequent both in the leucocytes and in the diplococci. Many leucocytes, even those devoid of diplococci, show nuclear fragmentation and vacuolization, while the markedly degenerated diplococci are commonly intracellular. The number of diplococci in the leucocytes is as variable in the guinea-pig as it is in man. Occasionally one finds that the injected diplococci have undergone agglutination in the peritoneal fluid. Where the injected diplococci were quite uniformly distributed in the salt suspension, cover slip preparations from the inflammatory exudate show the micro-organisms to be in smaller and larger clumps, in which condition they may be taken up by phagocytes.

Since in cerebro-spinal meningitis in man cultivation experiments may fail to yield the diplococcus although the microscope shows it to be present, attention may be called to similar failures with guinea-pigs. In the case of these animals the period of survival after inoculation affects the result, since, if the autopsies are made soon after death, those pigs succumbing earliest give the greater number of successful cultivations. But if the autopsies are delayed and par-

tial decomposition of the body has already set in, or if the dead pigs have been kept for many hours in the refrigerator, the chances of successful cultivation of the diplococcus are greatly reduced. I have noticed that the diplococci taken from pigs dead some hours, which have not grown on planting on a good medium, show irregularities in staining indicative of beginning dissolution; while a pig which had died at about the same time from a corresponding injection of the diplococcus, but which had been kept in the refrigerator during the same number of hours (six to eight), showed the diplococci in a better state of preservation, although they too may not have grown in the tubes. In the first instance, the diplococci have succumbed, probably, to the combined action of inflammatory exudate and the products of decomposition; and in the second, they were probably killed by the low temperature (*vide supra*). Should the former pigs remain somewhat longer at the fairly warm room temperature, the diplococci may disappear entirely *post mortem*. Hence, failure to grow upon artificial culture media may depend upon changes suffered by the diplococci in the infected body, or in the body after death. I may anticipate, in this place, an observation made upon the medulla oblongata of a monkey which succumbed to an intra-spinal injection of the diplococcus. The medulla was covered with a recent purulent exudate from which diplococci were easily cultivated. Two portions were excised of which one was placed at 37° C. and the other at 2° C. over night. Neither of these now gave growths, and the cover slip preparations which originally showed numerous diplococci now showed many fewer in the exudate from the section of tissue placed in the thermostat; no such diminution occurred in the cooled tissue.

In view of the manner in which *Diplococcus intracellularis* acts in causing death of guinea-pigs, it may be assumed that its effects are produced by a poison liberated from the bacterial bodies, probably through disintegration, and not to a secreted extracellular poison. Multiplication on the part of the diplococci is not at all essential to the production of the peculiar symptoms and lesions. Even though multiplication of the virulent diplococci does take place, it is still likely that the toxic action is caused by the diplococci which have suffered dissolution. That this is the correct

interpretation is shown by the toxic action (1) of cultures killed by heat, and (2) of the autolyzed fluids—autolysates—of cultures. As regards the autolysates, they can readily be shown to be lethal, by intraperitoneal injection, in mice and small guinea-pigs, of which the latter are the more sensitive. The autolysate prepared by suspending the growth from a pint Blake bottle in 12 c.c. of salt solution will, after filtration through porcelain or centrifugalization till clear, cause death in twenty-four hours in doses of 0.5 to 1.0 c.c. It seems as if the filtered extracts had lost something of their toxicity as compared with the centrifugated ones. Exudates obtained from the peritoneal cavity of guinea-pigs, in which many diplococci had been dissolved, also proved to be toxic for mice. Heating the autolysate to 65° C., at which temperature the enzyme is reduced in activity, does not reduce materially the toxicity; hence, the poison is probably independent of the enzyme. The following tabulation shows the comparative effects of living and dead cultures and auto-

Weight of Pig in Grams.	Substance Injected.	Result.	Post-mortem Appearances.
187	0.5 c.c. susp. living cocci.	Died during night.	8 c.c. turbid fluid in peritoneum; typical lesions. Large numbers of diplococci; some phagocytosis.
150	0.5 c.c. susp. heated to 60° C.	Died during night.	4 c.c. haemoglobin-tinted fluid. Mottled adrenals. Oedema pancreas. No phagocytes or diplococci.
190	1.0 c.c. susp. heated to 60° C.	Survived.	
182	2.0 c.c. susp. heated to 60° C.	Survived.	
260	3.0 c.c. susp. heated to 65° C.	Died during night.	5 c.c. haemoglobin-tinted fluid; typical lesions; multiple haem. in liver; some leucocytes; few fragments; swollen cocci.
152	1.0 c.c. autolysate.	Died during night.	4 c.c. slightly turbid exudate in peritoneum; mottled adrenals; some leucocytes.
182	1.0 c.c. autolysate heated to 65° C.	Died during night.	4 c.c. clear fluid in peritoneum; red adrenals; no leucocytes in peritoneum, but some in omentum.

lysates. All the injections were made into the peritoneal cavity at four P. M.

The fact that this table especially brings out is that heating the diplococci to 60° C. or slightly above reduces their toxicity, while the toxicity of the autolysate is not materially reduced by the temperature. Upon what changes in the diplococci produced by this temperature the loss depends is not shown by this experiment, but perhaps the reduction of enzymotic power, which begins to take place about 60° C. may partially explain the effect. Since the toxic action of the diplococcus depends upon disintegration, reduction in the rapidity of dissolution might easily affect the result. Relation between the weight of the pig, as influencing susceptibility, is also shown to play a part, but one easy to be overcome by increasing the dose of the diplococcus.

In order to ascertain how important viability of the diplococcus is in bringing about the lethal effects a suspension in Ringer's fluid of which the fatal dose had been determined was kept at 2° C. until growth could no longer be obtained. It was assumed that the toxin suffers less injury when the cocci are killed at low than at the higher temperatures. The lethal dose went up from 0.1 c.c. to 0.2 c.c. of the suspension. A second observation bearing on this point was unwittingly made in the course of some experiments with immune sera. A suspension of the diplococcus in Ringer's fluid of which 0.1 c.c. was fatal in 12 to 14 hours in the preliminary test was found, the next day, to be without power to cause death in the control animals and, of course, in the immunized pigs. A large volume of the suspension was transplanted to sheep-serum agar but no growth was obtained. The assumption that multiplication of the injected diplococci takes place during the first hours after inoculation would seem to have more probability than the counter assumption that deterioration of the poison sometimes takes place rapidly from the action of unknown causes.

The most striking lesions in inoculated guinea-pigs occur in the peritoneal cavity. Unless the lethal effect is prolonged beyond one or two days, fluid exudates are obtained, and more or less pus and fibrin cover the anterior surface of the liver, and the surfaces of

the rolled-up omentum. Even with considerable purulent deposit over the liver, the fluid may be nearly clear. The pleural cavities frequently contain an excessive quantity of clear serum, and the lymphatic glands generally are swollen and congested. The main visceral lesions met with are (1) vivid congestion or hæmorrhages of the adrenal glands; (2) hæmorrhages into the mesentery, central tendon of the diaphragm and serosa of the abdominal walls and intestine; (3) gelatinous œdema of the pancreas and peri-pancreatic tissues. A detailed description of these lesions is not merited; but exception may be made of the hæmorrhages in the mesentery. This exception appears justified by the not uncommon occurrence of hæmorrhages into the skin in cerebro-spinal meningitis in man.

The hæmorrhages in the peritoneum in the guinea-pigs are distributed with much regularity. The most common places of occurrence are the mesentery of the ascending colon, below the pancreas, which has an almost circular form derived from the curved loops of intestine, the mesentery of the cæcum, and the central tendon of the diaphragm. Hæmorrhages occur less frequently in other parts of the serous membrane. The mesentery is stretched over bottle-tops, fixed in Zenker's fluid, and stained in hæmatoxylin and eosin. The preparations are thin enough to permit the examination to be made with the one-twelfth inch immersion lens. The hæmorrhages are strictly focal. They arise from capillaries and small veins; and they are common at the point of junction of several veins and capillaries. The extravasation is, usually, from one point of the vessel, but the infiltration of the vascular sheath and adjacent tissue with red corpuscles may be quite extensive. Sometimes only a few corpuscles escape. The vessels from which the blood escapes are not thrombosed; there is often no evidence of marked stasis; judging from the few polymorphonuclear leucocytes adjacent to the escaped corpuscles, inflammation is not a prominent feature. Often no change in the vascular walls at the place of escape of corpuscles can be made out; sometimes the endothelium is massed at the point of apparent escape; rarely is the vessel dilated above or below the point; but in some instances the endothelium has been displaced outwards by the escaping corpuscles.

or even appears to be deficient. The last appearance is similar to that which Flexner and Noguchi<sup>11</sup> described in rattle-snake venom poisoning as caused by "hæmorrhagin," an endotheliolysin contained in that venom.

It has been noted that the toxic autolysate carries all the toxic bodies contained in the diplococci. It is, however, doubtful whether all of them pass equally readily through Berkefeld filters. Larger doses of the filtered "extract" are required than of the centrifugalized ones to produce lethal results; and the exudates caused by the former may not be attended by accumulations of fibrin and pus. Possibly the small fragments of bacterial bodies, not separated by the centrifuge, bring out the richer emigration of leucocytes. Hæmorrhages have, however, been hardly ever noted among the lesions caused by the filtrate.

The great capacity which the body displays for ridding itself of the diplococci, as shown by the enormous numbers of them which disappear from the peritoneal cavity in a few hours, could be readily accounted for by the inherent tendency to self-digestion which the diplococci exhibit. Any mechanism which will either kill the micro-organisms outright, or so reduce their vitality as to bring them under the injurious influence of their own enzyme will suffice to bring about their dissolution. It has already been pointed out that phagocytosis is not essential to the removal of the diplococci from the peritoneum and the reason for this is now clear. It is relatively uncommon to find, in human infections, any number of the diplococci circulating with the blood, and in guinea-pigs, mice and monkeys, the diplococci are present in the blood for a brief relative period only. From these observations the fact might be deduced that the diplococcus does not thrive in the blood serum. David Davis<sup>12</sup> has observed the diplococcus to grow in defibrinated blood of certain normal individuals, but he has also ascertained that normal serum is bactericidal for the diplococcus, and this destructive property is diminished but not wholly removed by heating the serum to 60° C. for thirty minutes. Hence, the question arises whether in the guinea-pig this mechanism of serum-lysis or any

<sup>11</sup> *Univ. Penna. Med. Bulletin*, 1902, xv, 345.

<sup>12</sup> *Jour. of Infectious Diseases*, 1905, ii, 602.

allied phenomenon is employed naturally in removing the diplococci from the peritoneal cavity. An answer to this question may help to explain the bactericidal phenomena which take place in the peritoneal cavity of these animals.

I have made experiments on the dissolving power of the blood-

Two guinea-pigs received intraperitoneal injections of *Diplococcus* "654" heated previously to 65° C. for thirty minutes. They survived about eight hours. The clear peritoneal exudate was collected: it contained very few leucocytes and many shadowy diplococci. Centrifugalization until clear. Two series of tests were made as follows: fresh guinea-pig serum and the fresh exudate with large and small amounts of living diplococci; serum and exudate heated to 60° C. with the same amounts of living cultures. The tabulation gives the result.

#### SERIES I.

	Immediate Culture.	Culture after 24 Hours.	Culture after 48 Hours.
Serum 2 c.c. emulsion cocci 0.1 c.c.	Abundant growth.	Reduction in growth.	Many separate colonies.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Idem.	Idem.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
	Immediate Cover-slips	Cover-slips after 24 Hours.	Cover-slips after 48 Hours.
Serum 2 c.c. emulsion cocci 0.1 c.c.	Many well stained cocci.	Small number of cocci remain.	Increased number of cocci.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Reduction less than previous one; swollen cocci visible.	Greatly increased number of cocci.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only shadows of cocci remain.	No cocci.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only detritus.	Detritus.

Series II was a repetition of Series I, except that 0.5 c.c. of the emulsion of the diplococcus was employed. The striking feature was the complete destruction of the organisms by the unheated exudate; the apparent complete destruction at the end of the first twenty-four hours by the exudate heated to 60° C. and the re-appearance of a small number of colonies from this tube at the end of the second twenty-four-hour period. The serum tubes gave abundant growth in all the transplantations. Cover-slip preparations showed reduction in numbers of diplococci in all the tubes, the reduction being less marked in the tubes of serum than of the exudates.

serum and the peritoneal exudate, from guinea-pigs on the diplococcus. The serum is collected in a sterile manner; the peritoneal exudate is freed from cells by centrifugalization. Both fluids possess digestive power for the diplococci, but in very different degrees. Mixed with fresh cultures considerable numbers of the diplococci can be destroyed and dissolved. In the case of the exudate, the toxicity, as might be expected, is increased by this digestion. The serum and exudate differ not only in respect to the number of diplococci which they destroy in their natural condition, but they also differ as regards the effect of heat on the bactericidal power. Heating to 60° C. for thirty minutes robs the serum of much more of its power to destroy the diplococci than the exudate.

Practically the same result as the preceding has been obtained with exudates derived from guinea-pigs succumbing to living cultures. The power to destroy diplococci is not lost by the exudate after several weeks keeping at 2° C. In the course of the destruction of the diplococcus by the exudate, I have repeatedly noticed the enlarged or swollen, deep-staining diplococci already mentioned of which it has been suggested that they may be more enduring forms of the micro-organism. In safranin-stained preparations these diplococci stand out as sharply defined, highly refractive, deep pink bodies. They are the last morphological elements to disappear from the fluids; and when they can no longer be detected the cultures prepared from the fluids remain, as a rule, sterile. Fresh guinea-pig serum, and to a less extent the heated serum, can destroy all the diplococci added if the number is not too great. There appears to be variations in this power in different normal animals.

The question upon what ingredients the difference in action of serum and inflammatory exudate depends, arises naturally. Perhaps the most important difference in composition would arise from the products of diplococcal disintegration and of leucocyte destruction in the exudate as compared with the serum. We have already seen that the products of diplococcal dissolution are injurious to living diplococci only when they are highly concentrated, and hence it seems as if in the case under consideration they could be left out of account. My own opinion is that the destroyed leucocytes yield to the exudate an injurious substance which acts upon the diplococci



in such a manner as to bring them under the dissolvent action of their own enzyme. Since, also, the fresh and heated (60° C.) exudate is digestive upon diplococci heated to 65° C. and 70° C., it must itself possess dissolvent power. This latter power is probably not complementary but rather proteolytic in nature. Whether the diplococci are subject in high degree to the complementary form of bacteriolysis is not known; but the power exerted by serum heated to 60° C. would rather indicate that another kind of destruction than this ordinarily takes place. What this is can be surmised from the general tendency to dissolution exhibited by the diplococcus. It is necessary only that fresh or heated serum should injure the diplococcus in order that its intracellular bacterial enzymotic power should come into action. In order to explain all the appearances met with in the peritoneal cavity of guinea-pigs infected with *Diplococcus intracellularis*, the various properties of the diplococcus, plasma, and inflammatory exudate, have to be taken into accurate account.



## EXPERIMENTAL CEREBRO-SPINAL MENINGITIS IN MONKEYS.

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### PLATES II-V.

At the beginning of my study of *Diplococcus intracellularis* I had the plan of using monkeys to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. My study of the intraperitoneal injections of guinea-pigs encouraged me to believe that the injection of suitable cultures of the *intracellularis* into the spinal canal of monkeys would lead to the production of an acute inflammation, the symptoms and lesions of which might bear resemblance to those occurring in the natural infection in human subjects. I was not mistaken in this belief. Some of my earliest experiments were made with monkeys. The cultures were introduced into the spinal canal by means of lumbar puncture. The injection was made commonly in the canal at the level of the third lumbar space. This space is not below the level of the cord in many monkeys, but I have never had paralysis follow my inoculations. I endeavored to secure a flow of spinal fluid through the needle before injecting the culture, for, otherwise, doubts whether the injection was carried into the canal or the surrounding muscles might arise. Not infrequently I failed to obtain this flow of fluid even when I felt reasonably certain that the needle was in the canal. In endeavoring to remove this source of doubt I introduced, in a few instances, the needle into the fourth and fifth lumbar spaces, without causing, in a single instance, paralysis. It sometimes happened that fluid could be obtained at these higher levels when it was not secured at the lower ones. Small monkeys, as a rule, seem not to have any considerable quantity of free fluid in the spinal canal. In this respect, this class of animals differs greatly from man. The amount of fluid which is obtainable by lumbar puncture from the monkey

is subject to wide variation in the same species, and in different species. The amount which may be secured from an animal on successive days also differs greatly. In some cases, a few drops may be secured after patient waiting for five or ten minutes; in other cases, the flow sets in immediately and one or two cubic centimeters are rapidly obtained. As will appear below, the quantity of fluid is increased at certain stages of the inflammatory process, to diminish or disappear wholly at others.

In cases in which no spinal fluid has been obtained preceding the injection of the culture, a successful injection can be assumed if the monkey develops symptoms of illness within a few hours of the inoculation, or if a subsequent puncture of the spinal canal yields fluid containing the diplococci and exuded inflammatory cells. I have found that the injection of relatively large quantities of living diplococci into the muscles about the spinal column, or into the muscles and subcutaneous tissues of other parts of the body, produces only trifling effects. There appears at most some local swelling, and the animals may refuse food for a few hours. The tumefaction quickly disappears and the appetite returns. There is a parallel in this respect between the relative susceptibility of the guinea-pig to intraperitoneal inoculation and the high degree of refractoriness which it displays toward subcutaneous injections of the diplococcus. Not a few cultures of the diplococcus have failed to produce symptoms or lesions when injected into the spinal canal of monkeys. Virulence plays an important part in these injections. In some instances the degree of virulence as exhibited in monkeys and guinea-pigs was similar. Cultures which were active against guinea-pigs were active in monkeys; a culture of low virulence for the former was little pathogenic for the latter animals. This parallelism was well shown by a culture "Smith," which retained its virulence through several months of artificial cultivation. When its power to infect guinea-pigs was finally greatly diminished, it had lost to a large degree its power to cause serious lesions in monkeys. In the experiments upon monkeys to be related, suitable cultures for inoculation were, as a rule, chosen by testing them in advance upon small guinea-pigs.

At the time my experiments were begun there was, as far as I

was aware, only one report in the literature upon the use of monkeys to produce cerebro-spinal meningitis by injecting cultures of *Diplococcus intracellularis* into the central nervous system. Bettencourt and Franca<sup>1</sup> attempted without success to infect monkeys. They trephined one monkey and introduced the culture beneath the dura mater; they injected in two other monkeys cultures into the spinal canal. In still another monkey they attempted to infect the animal by rubbing the nasal mucous membrane with cotton moistened with a culture of the intracellularis. The successful experiments of Von Lingelsheim and Leuchs<sup>2</sup> were published after my first communication on this subject had appeared.<sup>3</sup> Weichselbaum<sup>4</sup> succeeded by subdural inoculation in three instances in dogs in producing acute pachy- and leptomeningitis associated with encephalitis; Councilman, Mallory and Wright<sup>5</sup> successfully produced acute meningitis in a goat by injecting a bouillon suspension of the diplococcus into the spinal canal. Albrecht and Ghon,<sup>6</sup> and Bettencourt and Franca<sup>7</sup> failed to confirm this result in their experiments upon goats.

The monkey offered itself as a suitable animal for the inoculation experiments because of its relationship to man; the fact of its upright carriage, which would permit comparison with man in respect to the distribution of the lesions, and because of the ease and safety with which repeated lumbar puncture could be made in following the course of the disease. The protocols of certain successful experiments will be given.

*Monkey No. 1. Macacus Nemestrinus.*—April 12, 1905, 10:30 A. M. A moderately large monkey was etherized,<sup>8</sup> after which a needle was introduced into

<sup>1</sup>Über die Meningitis Cerebro-spinalis Epidemica und ihren spezifischen Erreger. *Zeit. f. Hyg. u. Infectiouskrank.*, 1904, xlv, 463.

<sup>2</sup>Tierversuche mit dem *Diplococcus intracellularis* (Meinigococcus), *Klin. Jahrbuch*, 1906, xv, 489.

<sup>3</sup>Experimental Cerebro-spinal Meningitis and its Serum Treatment, *Jour. Amer. Med. Assoc.*, 1906, xlvii, 560.

<sup>4</sup>*Fortschritte der Medicin*, 1887, v, 622.

<sup>5</sup>Epidemic Cerebro-spinal Meningitis and its relation to other forms of Meningitis, Boston, 1898.

<sup>6</sup>*Wien. klin. Woch.*, 1901, xiv, 984.

<sup>7</sup>*Op. cit.*, page 500.

<sup>8</sup>In later experiments ether was not used, as the operation gave little pain.

the spinal canal. Clear fluid escaped from the needle. A culture eighteen hours old of *Diplococcus intracellularis*, in its first generations on Loeffler's serum, suspended in salt solution, was injected. The monkey quickly recovered from the operation. Six hours later it appeared to be sick; at 6 P. M., it no longer sat on its perch; 9 P. M., still on bottom of cage, very sick, head down. On being disturbed, it moved slowly. Death during the night. Survived injection probably eighteen to twenty hours.

Autopsy 9 A. M., April 13. The internal organs, except the nervous system, show no striking lesions. Brain and Spinal Cord.—No marked excess of fluid in the meninges. The convex surface of the brain is greatly congested, and punctiform hemorrhages exist in the pia-arachnoid and the superficial portions of the cortex. No definite exudate can be made out on the convex surface of the convolutions, although the pia of the sulci appears white and is slightly thickened (probably an old condition). A focus of softening or an abscess, the size of a large pea, occurs to the right of the superior longitudinal sinus in the convolutions anterior to the motor area, and just beneath the membranes. The base of the brain is covered with an opaque exudate, white in color, which extends over the medulla and, anteriorly, to the optic commissure. The lumbar and thoracic portions of the spinal cord are covered, chiefly posteriorly, with an opaque white exudate. The cerebral ventricles contain an increased amount of fluid of turbid appearance.

The diplococcus was recovered in cultures from the exudates of the spinal cord and base of the brain, and from the blood of the heart. Cover-glass preparations from the exudates show that (1) the pia of the convexity contains many polymorphonuclear leucocytes and few diplococci; (2) the pia covering the medulla contains large numbers of leucocytes and intracellular cocci, and a small number of extra-cellular cocci; (3) the ventricles contain desquamated ependymal epithelium, many leucocytes, and few diplococci; and (4) the focal lesion in the convexity of the brain consists of a collection of polymorphonuclear leucocytes containing very large numbers of typical diplococci. Few or no diplococci occur outside of cells in this focus (Plate II, Fig. 1).

Sections of the brain and cord show an abundant emigration of polymorphonuclear leucocytes into the leptomeninges chiefly. The pia-arachnoid of the convexity contains a richer exudate than was evident to the naked eye; but the exudate covering the base of the brain is present in much the greater amount. It consists chiefly of leucocytes; fibrin occurs in very fine strands and in small amount only. The ventricular fluid contains many leucocytes and no fibrin. A striking feature of the inflammation is the invasion from the pia-arachnoid of the superficial portion of the cortex directly, and of removed parts at considerable depths indirectly along the sheaths of the blood vessels. The vascular sheaths and the perivascular lymphatic spaces are richly infiltrated with polymorphonuclear leucocytes from which locations the surrounding brain tissue is being invaded by emigrating leucocytes. Sections through the filum terminale show the leucocytes to surround the cord in the pia, a slight invasion along the septa into the nervous tissue, and thick perivascular emigration about the deepest vessels of the dura mater.

This successful experiments was obtained by the use of a recent

culture isolated from the spinal fluid of a child "Kepp." The fluid was quite opaque from the many leucocytes present and it contained a moderate number of the diplococcus, both within leucocytes and free in the fluid. A second monkey of the same species and about the same size as the first was inoculated with one cubic centimeter of the sedimented exudate. Immediately after the injection, the monkey became rigid and the head was retracted; recovery took place in a short time. In spite of the fact that no fluid escaped from the needle, no doubt was entertained that the injection entered the spinal canal. However, no symptoms of disease developed from this injection.

*Monkey No. 3. Small Macacus rhesus.*—April 15, 1905, 9 A. M. Inoculated by lumbar puncture with two loops suspended in salt solution of "Bingley" culture of the diplococcus from spinal fluid. 5 P. M., animal sick; sits on bottom of cage. 9 P. M., very sick; crouches on bottom of cage with head depressed; moves slowly on being disturbed. April 16, 9 A. M., still alive; head down almost to level of the floor; a little later, the monkey is lying on one side and is passing through a convulsion. The convulsive seizures follow each other at short intervals and are excited by sudden noise or by contact. 3 P. M., convulsions still occurring; lumbar puncture yields a small quantity of bloody fluid which on microscopical examination shows many polynuclear leucocytes containing typical diplococci. 10 P. M., convulsions continue. Animal died during the night; probably survived about forty-three hours.

Autopsy, 9 A. M., April 17. No visible lesions of the internal organs except the central nervous system. The convex surface of the brain shows great injection of the pial vessels; and the pia-arachnoid contains a gelatinous fluid exudate. Purulent exudate is visible surrounding the infundibulum only. The fluid in the ventricles is slightly increased. No visible exudate covers the spinal cord. The cultures made from different portions of the brain and cord remained sterile. Sections of the spinal cord show a small quantity of purulent exudate in the meninges; those from the brain show a richer leucocytic infiltration of the pia-arachnoid, and the invasion of the choroid plexus of the lateral ventricles. The meninges of the sulci are especially infiltrated. From the superficial meninges, and from the intra-cortical vessels, the brain substance has been infiltrated with many leucocytes.

As regards this experiment I wish to point out that when the inoculated monkey survives through the second day, the quantity of exudate may not be very considerable, and the diplococci, if still present, may fail to grow on a suitable culture medium.

*Monkey No. 7. South American; Genus Cebus.*—May 19, 1905, 3 P. M. One cubic centimeter of a suspension of spinal fluid culture "Goldman" injected. This South American species is considerably smaller than the *Macacus* monkeys employed. It was found dead at 7 A. M., May 20, but evidently had been dead

only a short time. Probably survived fifteen hours. The autopsy showed a small amount of exudate over the spinal cord in the lumbar region, turbid fluid along the base of the brain to the optic commissure, vivid injection of the pia of the convexity, and an increased quantity of turbid fluid in the cerebral ventricles. Cultures were positive. Smear preparations from different parts of the brain and cord show the leucocytic exudate to be general, and a fairly large number of diplococci, chiefly intracellular, to be present. Many of the diplococci within leucocytes are swollen or otherwise degenerating. The preparations made by smearing bits of the nasal mucosa on slides show a considerable number of polymorphonuclear leucocytes among the high epithelium and mucus. *A small number of the leucocytes contain many diplococci morphologically like those of the exudate in the brain, and presenting the same degenerations.*

A study of the brain and cord in sections shows the inflammatory lesions over the convexity to be more pronounced than was evident to the naked eye. The pia is everywhere invaded by polynuclear cells, and the exudations form thick, wedge-shaped infiltrations between the convolutions. A fine network of fibrin unites the leucocytes. The invasion of the cortex is a marked feature of the lesions. The leucocytes have passed into the brain tissue from the surface and along the sheaths of the vessels (Plate II, Fig. 2). An intermediate zone of non-infiltrated tissue exists between the two layers. The invasion of leucocytes into the brain stops rather abruptly a centimeter or so from the surface, and the deeper vascular sheaths are devoid of these cells. Among the leucocytes which have emigrated into the cerebral tissue are a small number of eosinophilic cells. A section through the medulla shows less exudate than some parts of the cortex. The deeper parts of the medulla have also been invaded from the vascular sheaths. The lateral ventricle has been invaded along the choroid plexus, and an accumulation of leucocytes occurs below the epithelium, which is partly deficient, at one side of the ventricle. The thoracic region of the spinal cord is relatively free from exudate.

A spinal root ganglion included in a section of the spinal cord is surrounded, beneath its fibrous capsule, by a collar of leucocytes which are penetrating among the nerve cells (Plate III, Fig. 3). Sections of the optic nerve show leucocytic invasion. Diplococci are numerous in the leucocytes in the pia-arachnoid. They cannot be found with certainty in the leucocytes or free in the brain tissue.

This experiment serves to show that in a brief period of fourteen or fifteen hours advanced and deep lesions can be produced in the brain and its membranes by *Diplococcus intracellularis*. The experiment also indicates that the inflammatory reaction may be more active in the brain than in the spinal cord. The inflammatory exudate resembles in character that present in acute cases of epidemic meningitis in human subjects. Emphasis should, perhaps, be laid upon the findings in the nose and in the spinal ganglion. It is usual to find in the monkeys succumbing to the experimental infections, evidences of inflammation of the nasal mucosa. Smear preparations from the superior mucous membrane often show a



variable number of leucocytes enclosing diplococci presenting the morphology and staining properties and the degenerations of the diplococci in the brain and cord. The inflammation and purulent infiltration of the spinal ganglion is interesting in relation to the similar finding in cases of cerebro-spinal meningitis in man by Councilman, Mallory and Wright.<sup>9</sup>

*Monkey No. 10. Macacus rhesus.*—May 26, 1905. At 11 A. M. one half agar culture "Cohn" suspended in salt solution was injected into the spinal canal. Fluid escaped from the inserted needle. 5 P. M., animal very sick; living at 9 P. M. Found dead at 6 A. M., May 27. Probably survived fifteen hours.

Autopsy: The meninges of the inoculated portion of the cord show minute hæmorrhages and cloudy exudate. The meninges of the medulla contain an increased quantity of cloudy fluid. The pia covering the convex surface of the brain is hyperæmic, especially over the occipital lobes, which present an almost uniformly reddish tint; while the pia and the adjacent brain substance are everywhere beset with minute hæmorrhages. Cultures from the lumbar portion of the cord, third ventricle, and pia over the medulla all give pure growths of the diplococcus. No growths are obtained from the cortical meninges and the lateral ventricle. The furthestmost portion of the dura mater, extending beyond the olfactory lobes into the nose, is covered with an inflammatory exudate in which polymorphonuclear leucocytes carrying biscuit shaped Gram-negative diplococci exactly resembling in form, preservation and staining, those present in the meninges of the brain and cord occur. Smear-preparations from the turbinated bones show very few leucocytes and no diplococci. Cultures made from the mucus of the turbinated mucous membrane did not yield the diplococcus.

The sections of the brain show a moderate inflammatory cedema affecting the pia-arachnoid of the base and the convexity. The especial pathological conditions to be emphasized are (a) the great congestion of the pial blood-vessels and the extravasation of red corpuscles; (b) the direct inflammatory invasion of the superficial cortex from the pia which is associated with rarification of the brain tissue; (c) the profound changes in the blood-vessels of the brain proper and the degeneration and leucocytic invasion of the surrounding brain tissue (Plate III, Fig. 4). In some parts of the cortex, adjacent to the sulci, the leucocytic accumulation is so rich as to simulate abscess formation; no softening has, however, taken place. The emigration of leucocytes from the intracortical vessels was active at the time of fixation of the tissues. The perivascular lymphatics contain fibrin and coalesced red-corpuscles, and the blood-vessels themselves are occluded, in places, by coalesced (agglutinated) red corpuscular and by fibrinous thrombi. The punctiform hæmorrhages in the brain tissue arise from these injured and occluded vessels. *Sections of the dura mater extending beyond the olfactory lobes show a richer accumulation of leucocytes than elsewhere in the membrane itself and an invasion of the substance of the membrane.*

Typical diplococci occur within leucocytes in the meningeal exudation and

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<sup>9</sup> *Op. cit.*, p. 114.

they are moderately numerous. They exist also in the leucocytes in the brain tissue, adjacent to the blood-vessels from which the leucocytes have emigrated. No diplococci are found in the brain tissue outside of cells.

A lengthy discussion of this experiment is not called for since it carries its own explanation. Attention may, perhaps, be directed again to the co-incident effects produced by the diplococcus upon the membranes and the nervous tissues. The wide involvement of the blood vessels is a significant fact in the pathology of this experimental disease; and the evidence of diffusion of the poison from the blood vessels of the brain or from the peri-vascular lymphatics into the brain tissue is made strong by this experiment. The agglutinative red corpuscular thrombi constitute a novel feature of the pathological condition. The rich assemblage of leucocytes surrounding the olfactory lobes and extending into the substance of the dura mater seems to me not wholly without significance in view of the possibility of infection of the nasal passages with diplococci from the brain.

In view of the importance of this condition for explaining the appearance at times, of *Diplococcus intracellularis* in the nasal and pharyngeal cavities in human subjects of cerebro-spinal meningitis, I gave especial attention to the study of the olfactory nerves and the nasal passages in several monkeys. It may, in the first place, be stated that the turbinate and septal mucous membrane will be found to be vividly congested in all the animals which succumb to the acute infection. If smear preparations from the mucous covering the inflamed membranes, or, better, from bits of the mucous membrane snipped off with scissors be examined, a variable but increased number of polynuclear leucocytes will be seen. If the leucocytes are compared with those present in the meninges about the base of the brain, they will be found in the same condition of preservation or degeneration as the latter. Moreover, in those cases in which diplococci are still demonstrable in the leucocytes in the brain, a certain number, sometimes relatively many, of the leucocytes in the nose contain Gram-negative diplococci resembling in all external features the former micro-organisms. If the dura mater beginning at the olfactory bulbs, surrounding the olfactory nerves, and extending through the cribiform plate into the nose, be carefully removed with the adjacent

portion of the ethmoid bone and olfactory mucous membrane, fixed in Zenker's fluid, sectioned longitudinally, stained in hæmatoxylin or methylene blue and eosin, and examined microscopically, the passage of leucocytes from the brain cavity into this membrane and about the olfactory nerves towards the ethmoid, can be traced. The blood-vessels of the adjacent olfactory mucous membrane are seen to be dilated and leucocytes to be passing into the layer of columnar epithelium of the surface.

The abundant lymphatics of the mucous membrane are in communication with the lymphatic spaces which enclose the branches of the olfactory nerves, and these spaces again communicate with the subdural and subarachnoid spaces of the cranium, so that the lymphatics of the nasal mucous membrane can be injected, according to Schwalbe and Key and Retzius, from the cranial cavity.

I have not succeeded, although I have made several attempts, in cultivating *Diplococcus intracellularis* from the nasal mucosa of these monkeys. I realize, of course, that it is highly desirable and very important to bring this final proof of the passage of the diplococci from the cranial cavity into the nose. The difficulties which surround the demonstration are considerable, for I think it probable that the diplococci do not long remain viable in the nose in monkeys. I conceive the conditions there to be, possibly, even more unfavorable to them than in the meninges where, indeed, they tend rather to disintegrate than to multiply. The conditions in man are, of course, very different and far more favorable to the existence of the diplococci. But in spite of the incompleteness of the proof in the case of the monkeys, I would still urge that attention be given to this possible mode of infection of the nasal and pharyngeal passages in human subjects.

The next experiment to be described brings out a different point, namely, that of a fatal issue, after the lapse of thirty hours, with which is associated the gradual diminution and degeneration of the diplococcus, without any marked participation of leucocytes in the process, or production of striking pathological lesions. It recalls forcibly certain observations made on guinea-pigs.

*Monkey No. 12. South American; Genus Cebus.*—June 1, 1905, at 11 A. M., a small monkey was inoculated with one half agar slant culture "Smith"; fluid

flowed from the needle before injection. 12 M., lumbar puncture (= l. p.) 1 c.c. almost pure blood obtained; it showed on cover-slips (= c. s.) many free diplococci. 3:15 P. M., l. p. small quantity blood secured; c. s. many cocci free and very few in leucocytes. Animal not sick. 9 P. M., l. p. a few drops of slightly blood-tinted fluid obtained; c. s. many free diplococci and a small, although increased number of leucocytes containing diplococci. Animal less lively than before. June 2, 9 A. M., monkey is very sick; temperature sub-normal; head droops below arms; no convulsions on disturbance; l. p. a few drops of blood-tinted fluid secured; c. s. leucocytes increased, some containing diplococci part of which stain feebly; many free diplococci. 3 P. M., l. p. leucocytes still not numerous; intracellular and extracellular diplococci present, but the number is diminishing. Cultures from the fluids obtained at the punctures gave growths in every instance. About 4 P. M. the monkey showed general weakness and retraction of the head. Dr. Meltzer, who examined the animal, pronounced the latter condition not to be opisthotonus. Death at 5 P. M. On ice till next day.

Autopsy, June 3, 9 A. M. Neither the cord nor the brain show any marked lesions.

The pia of the cord is slightly injected; a small quantity of clear fluid is present in the pia of the medulla; the intraventricular fluid may be slightly increased. Cultures are negative from brain and cord and the heart.

Smear preparations from the meninges of cord and brain and the fluid of the ventricles show a small or moderate number of diplococci lying free, and almost no leucocytes whatever except in the spinal cord at the point of inoculation. A moderate number of the cells containing diplococci, many of which are degenerating, occur there. Elsewhere it is exceptional to find polynuclear cells containing diplococci. The failure of the diplococcus to grow in cultures made at autopsy does not signify, since the power to grow may be quickly lost at the temperature of the refrigerator. Smear preparations from the nose show no typical diplococci, while a few typical organisms are present in the smears from the pia about the olfactory lobes. The microscopical examination of sections of the spinal cord and brain bear out the naked eye appearances. The membranes show a slight but definite accumulation of leucocytes; there is no œdema, and the nervous tissue is free from appearances of degeneration or from leucocytic invasion. The leucocytes are, however, increased in numbers in the cortical vessels.

The next experiment records an instance of fatal effects, from a relatively small dose of the diplococcus, which was delayed until about sixty hours after injection. The gross lesions were slight; the microscopical ones were definite and interesting because the period of infection was long enough to permit of reaction on part of the fixed tissue cells.

*Monkey No. 18. Macacus rhesus.*—June 24, 1905, 10 A. M., injected one third culture "Smith." Symptoms of illness developed in the usual time and were pronounced during the first twenty-four hours. June 25, 12 M., lumbar puncture. The fluid obtained showed many leucocytes, but no diplococci. Cul-

ture negative. The animal did not fully recover and died about sixty hours after inoculation. The autopsy revealed congestion of the meninges, and a small excess of turbid fluid in the lateral ventricles. Cultures on sheep-serum agar were made from several parts of the nervous system. Growths were obtained on the second day, starting from the condensation water, in the tubes inoculated from the third ventricle and lumbar cord. Sections from the brain and cord show, first, a marked congestion of the veins which is greater in the meninges of the brain than of the cord. Second, a small degree of leucocytic exudation throughout the meninges, which in the brain is more abundant in the sulci than over the convolutions. But the most striking and, perhaps, important feature is the great increase of large monocular pial or connective-tissue cells which by reason of their number make up a large part of the exudate. These cells are larger than leucocytes, possess single vesicular nuclei excentrically placed, and pale and transparent protoplasm.

To be contrasted with the previous experiment is the next one in which the inoculated monkey survived hardly more than twelve hours, in the course of which period marked gross lesions appeared in the nervous system.

*Monkey No. 17. South American; Genus Cebus.*—June 22, 1905, 10 A. M., injected one half agar culture "Smith," twenty-four hours old. Fluid flowed from needle before injection. 9 P. M., monkey dying. Found dead at 6 A. M., June 23. The autopsy showed the entire spinal cord congested and infiltrated with turbid fluid. In the cerebral meninges smaller and larger hæmorrhagic foci were visible. The pia of the pons and medulla was vividly congested, and a fluid exudate occupied the meshes of the pia-arachnoid generally, and was most abundant over the medulla. The ventricles were unchanged, apparently. Cultures gave the following result: Abundant growth of the diplococcus from the lumbar cord and base of brain; a few discrete colonies from the cortex; no growth from the lateral ventricle. Smear preparations bore out the naked eye indications of the distribution of exudate and diplococci. Sections of the tissues show marked leucocytic infiltration of the membranes of the cord and brain, and a greater accumulation of cells in the basal membranes and the membranes of the sulci. The infiltration is wholly polymorpho-leucocytic. The most striking lesion, however, is the hæmorrhage which is focalized in the membranes and in the superficial brain matter—in the basal and cortical parts. The hæmorrhages in the brain tissue proper have arisen (*a*) by direct extension from the menigeal extravasations, and (*b*) from the vascular branches within the substance of the organ itself. They form elongated or circular foci, depending on the direction of the section. Another lesion of interest is an acute endarteritis which affects the larger and smaller arteries, chiefly at the base of the brain. A sub-intimal infiltration of cells consisting of polymorphonuclear leucocytes and mono-nuclear cells is seen. The vessels are rarely wholly or almost occluded by this accumulation of cells beneath the intact endothelium. Acute encephalitis was a prominent feature of this case.

This experiment, taken into account with others to be described, tends to show that the diplococci rise in the spinal canal to the

medulla, spread themselves over the base of the brain, extend into the cortical meninges, and lastly enter the ventricles. The inflammatory lesions follow in the wake of this extension and doubtless are directly due to the presence of the diplococcus. Attention should perhaps be directed to the hæmorrhages; and to the acute endarteritis mentioned in this protocol of which other examples will be described.

*Monkey No. 25. Macacus rhesus.*—June 21, 1906, 11 A. M. Inoculated with one agar culture "596." Fluid flowed from needle before injection. 2 P. M., animal appeared sick; lay on bottom of cage, but on being disturbed sat up. 6 P. M., depression increased. 12 P. M., still lying on bottom of cage, but not prone and rises to sitting posture when disturbed. June 28, 8 A. M., brighter; sits up. 12 M., attempted lumbar puncture. Animal resisted with considerable vigor making the puncture difficult. The struggles brought on sudden collapse from which partial recovery took place. Monkey lay on bottom of cage breathing very rapidly; drank water. 2 P. M., still very sick. From this time until 3:45 P. M., when death occurred the animal became weaker and developed intermittent convulsions. The autopsy showed no general visceral lesions. On exposing the spinal cord, it was found congested and covered with a thin, whitish exudate which was more abundant in the lumbar region than in the thoracic and cervical regions. The blood-vessels of the dura mater were injected; but the turgid pia vessels showed through this membrane. On removing the dura, the pia-arachnoid was seen to be vividly injected, the congestion affecting the main and smaller vascular branches and producing a remarkable picture of hyperæmia. There was no visible exudate over the convex surface of the brain. At the base, extending from medulla to optic commissure, there was a turbid fluid exudate. The ventricles contained turbid fluid, but were not dilated. The basal exudate followed the dura over the olfactory lobes into the ethmoid plate. The nasal mucosa was congested. Cover-slips showed the following: From the lumbar spinal cord many leucocytes and diplococci, the latter both free and in cells; from the thoracic and cervical regions of the cord, many leucocytes and fewer cocci; from the convexity—anterior and posterior—impression preparations indicated that few or no leucocytes collected in this region, and the diplococcus was almost wholly absent; from the medulla many leucocytes and extra- and intracellular diplococci; from the ventricles leucocytes and diplococci in small numbers; from the deepest part of the dura at the ethmoid bone many polynuclear leucocytes, but no typical examples of the diplococcus. Cultures from all the sources remained sterile. Since more than two days elapsed, owing to accidental circumstances, between the death of the monkey and the autopsy, it may well be that the failure of cultures was due to this cause. The same condition is capable of diminishing the number of diplococci which can be made out in smear-preparations. But the distribution of emigrated leucocytes showed clearly where the infection was severe, and where it was in its beginning stages. The sections of the tissues bear out the gross findings. They show the existence of great congestion of the oial veins, a small degree of leucocytic infiltration of the pia covering the con-

volutions, and somewhat greater accumulation of inflammatory cells in the sulci. The pia of the deeper parts of the sulci is hardly more than oedematous. The brain substance shows no leucocytic invasion, and the veins do not carry any marked excess of leucocytes. The lower spinal cord shows rather more emigration of leucocytes than the brain. The exudate is collected on the surface of the cord, upon and between the nerve roots (Plate IV, Fig. 5). Fibrin occurs as fine strands and in small quantity.

This experiment brings out several points, some of which have not been especially emphasized before. The effects of muscular exertion on the progress and termination of the infection should, perhaps, be remarked. Since in no other experiment was so great a degree of vascular congestion noted, I should be inclined to view this condition as one of the striking consequences of the muscular over-exertion. It is a short step from this assumption to supposing the marked oedema of the pia in the sulci to have followed the congestion. This view is rather borne out by the poverty of the oedematous fluid in emigrated cells. It has been pointed out on the basis of some of the earlier experiments, and the same fact is indicated by this experiment, that the injected diplococci tend, regularly, to be distributed upwards along the spinal cord and the base of the brain. The invasion of the pia of the convex and mesial surfaces of the brain takes place at a later period, and the infection of the ventricles also takes place later, either because the diplococci are delayed in reaching them, or because they resist infection longer. There is, moreover, relation between localization of the diplococci and the degree and extent of the exudation. Where the accumulation of inflammatory products is most marked the diplococci will, if still present at all, be most abundant. Finally, the growth of the diplococcus upon a suitable culture medium will be greatly influenced, if not wholly determined, by the conditions to which the infected body is subjected after death. While a temperature just above the freezing point will preserve the morphological elements of the body and even the body of the diplococcus, yet if it is maintained for many hours, it may render the diplococcus incapable of multiplication. Higher temperatures subject the body not only to autolytic and putrefactive processes which interfere with the pathological examination, but they deprive even more quickly than cold the diplococcus of power of growth and, under certain conditions, of staining properties as well.

The temperature at which the body is kept, and the elapsed period of time between death and the examination, have a direct bearing upon the cultivation of *Diplococcus intracellularis* from the infected body. I am of the opinion that these factors operate in much the same way in cases of human meningitis as in the case of the artificially infected monkeys and guinea-pigs. Certain discrepancies in the bacteriological studies of cases of meningitis in man may, in view of these considerations, receive an explanation.<sup>10</sup>

On account of its bearing upon this subject and as a transition to the next topic to be discussed, an experiment in which the influence of two extremes of temperature upon the diplococcus was tested, will be introduced here.

*Monkey No. 36. Macacus rhesus.*—October 24, 1906, 3:30 P. M. After clear fluid was obtained, one cubic centimeter of a turbid suspension of Culture "654" was injected. The injected quantity was the equivalent of two agar slants; the culture had proved itself of relatively low virulence for guinea-pigs. October 25, 10 A. M., monkey sick; sits on perch holding head in hands; easily disturbed. L. p. no fluid obtained; needle-point covered with exudate consisting of leucocytes crowded with diplococci staining sharply. The nuclei of a small number of cells contained cocci. October 26, 12 M., monkey brighter and more active. L. p. slightly cloudy fluid flowed at once from the needle. C. s. showed leucocytes in good numbers, some of which contained few and others very large numbers of cocci staining feebly. Many of the leucocytes had probably recently emigrated since they were of normal appearance with circular (horse-shoe), non-fragmented nuclei. October 27, monkey recovered. November 10, animal in excellent condition.

November 14, 4 P. M., l. p. gave fluid readily; it flowed from needle as if under some pressure. One cubic centimeter of suspension of coccus "596" injected. No immediate effect produced. 6 P. M., monkey less lively. November 15, 7 A. M., found dead; evidently died only a short time before. Autopsy. The spinal membranes were distended and on incision a small quantity of turbid fluid escaped. The cord appeared pale, probably because of the turbid exudate which covered it. The convex surfaces of the brain were moist; definite inflammatory exudate was not visible; the basal membranes, from the medulla to the optic commissure, were infiltrated and distended with a turbid exudate. The pituitary body was surrounded with exudate; the ventricles were not widely dilated. The nasal mucosa was pale. Of the other organs only the lungs showed a pathological condition, namely, congestion and œdema. Cultures from the brain and cord, made at 4 P. M., gave growths of the diplococcus; the heart and lungs gave no growth. At 5 P. M. two segments of the medulla, about 0.5 centimeter thick,

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<sup>10</sup> Westenhoeffer, Pathologisch-anatomische Ergebnisse der oberschlischen Genickstarreepidemie von 1905, *Klinisches Jahrbuch*, 1906, xv, 447.



were placed in sterile glass dishes which were enclosed in second dishes filled with cotton saturated with water. One was put at 37° C., the other at 2° C. At 10 A. M. next day, cover-slips and cultures were made; the latter gave no growth. C. s. showed the diplococci to have disappeared almost entirely from the tissue kept in the thermostat, the few remaining cocci being pale and ill-staining; and the diplococci from the second piece of tissue kept at 2° C. to be, perhaps, somewhat less numerous than in the control, but to stain sharply and well.

Histological examination of the central nervous system establishes the existence of a severe acute fibrino-purulent inflammation of the meninges of the cord and brain. The exudate in the cord is thicker over the posterior surface and it surrounds all the nerve roots; the dura mater is also invaded with pus cells. The exudate covering the medulla is abundant, while that covering the cortical surfaces is slighter in amount. The sulci show more exudate than the convexities. Two appearances should be emphasized: the great proliferation of endothelial cells of the pia, which cells mingle with the polynuclear leucocytes; and the diplococci, which are abundant in the exudate of the cord and medulla, innumerable in the exudate about the pituitary body and sparse in the exudate of the cortex and ventricles. The diplococci are, excepting those about the pituitary body, almost exclusively intracellular; and although a slight grade of encephalitis occurs, very few diplococci can be discovered in the leucocytes in the brain tissue. Thrombi of leucocytes occlude many veins.

The experiments reported thus far would seem to establish conclusively that the lesions and, to a certain extent, the symptoms of acute meningitis as they occur in man can be reproduced experimentally in monkeys. Since in the course of the experiments, the symptoms during life were observed incidentally only, the striking ones alone were recorded. Attention should be directed to the occurrence of nystagmus in a rhesus monkey which succumbed to inoculation. It should be stated, also, that not all the monkeys inoculated into the spinal canal succumb to the injections, next that after apparent recovery death may take place suddenly from causes which the post-mortem examination may fail to reveal, and finally that subcutaneous and intramuscular injections of large quantities of the diplococcus cause slight or no evident symptoms of disease. The cases of recovery will be illustrated by a few protocols. It should be noted that as recovery proceeds spinal puncture may show increasing cellular exudation into the canal and progressive degeneration of the diplococci. Cultures from the exudate at first positive become negative before all the diplococci disappear from it.

*Monkey No. 5. Macacus nemestrinus.*—May 15, 1905. This animal had been injected, April 12, 1905, with a purulent exudate without result (page 146). At

4:30 P. M. one cubic centimeter of a turbid suspension in salt solution of a diplococcus in its fifth generation from the spinal canal of case "Behren" was injected into the spinal canal. No symptoms had appeared by 9:30 P. M. May 16, 8 A. M., animal refused food and appeared sick. 11 A. M., had left perch and sat on bottom of cage. Appeared unable to lift himself on to the perch; while being observed two convulsions occurred. Placed on the perch, he held on with hands and feet and was in a state of unstable equilibrium. Made no effort to escape from the cage on being handled. 10 A. M., lumbar puncture; a small amount of bloody fluid containing a moderate number of leucocytes and extra- and intracellular diplococci obtained. 3:30 P. M., l. p.: a small quantity of purulent fluid secured. The greater number of cocci were within leucocytes, many of which showed fragmented nuclei. Cultures from the exudate positive for the diplococcus. 6 P. M., back on perch. May 17, 8 A. M., again on bottom of cage; looked very sick, but took a little milk. 10 A. M., l. p.: the number of diplococci had diminished; very few were outside of cells; those in leucocytes stained feebly. May 18, 10 A. M., l. p.: a few drops of blood-tinted fluid secured; c. s.: some polymorphonuclear leucocytes, increasing number of mononuclear leucocytes, and a very small number of leucocytes containing degenerated diplococci; a few extracellular diplococci still present. Cultures negative. This monkey recovered rapidly.

*Monkeys No. 19 and 20. Macacus rhesus.*—June 27, 1905, 10 A. M. Each of these monkeys was injected with one third culture "Smith." They became very sick during the afternoon, and lay on the bottom of the cage. 10 P. M., still very sick. June 28, 9 A. M., both animals looked brighter. 10 P. M., No. 20 seemed livelier than No. 19. June 28, 12 M., l. p.: both yielded a small quantity of turbid fluid. The fluid from No. 19 and No. 20 contained many leucocytes; the fluid from No. 19 contained typical intracellular diplococci in small numbers, while that from No. 20 contained none. A culture was made from each fluid: No. 19 gave one colony of the diplococcus, No. 20 gave none. Complete recovery quickly followed in both instances.

*Monkey No. 35. Macacus rhesus.*—July 10, 1906, 10 A. M. Given 1 c.c. of thin suspension of Diplococcus "596." Spinal fluid obtained with syringe. The only symptoms which developed were slight depression, and erection of the hair covering the body. July 11, 10 A. M., l. p.: a small amount of whitish fluid from which the cells quickly subsided, was obtained. C. s. showed a large number of agglomerated, polymorphonuclear leucocytes, and very few diplococci, chiefly contained in the cells. The few free cocci could easily have come from broken-up leucocytes. At this time, a second injection of 0.5 c.c. of a similar suspension of Diplococcus "596" was made; no result. Animal quickly recovered.

To insure a fatal outcome a diplococcus of known virulence in a sufficient dose must be injected. Individual differences in susceptibility occurred among the monkeys, but no monkey was wholly refractory to inoculation. As compared with the doses which probably determine infection in man those used to produce the experimental disease in monkeys are colossal. As an example of

refractoriness the next experiment will serve; it will show also how small the effect produced by subcutaneous and intramuscular injections of the diplococcus may be.

*Monkey No. 26. Macacus rhesus.*—October 28, 1905, 12:30 P. M. One "Smith" culture injected. 3:30 P. M., 1 p.: turbid fluid flowed from the needle. C. s. showed many diplococci and very few leucocytes. Some of the cocci grouped about the leucocytes. October 29, 12 M., 1 p. not successful; one half culture "Smith" injected (probably into muscles of back). October 30, 3:30 P. M., 1 p. unsuccessful. Injected two cultures "Smith" (probably into muscles of the back). 6 P. M., animal slightly depressed; off perch. October 31, A. M., animal active. 3 P. M., 1 p. yielded with difficulty a small amount of bloody fluid containing leucocytes and few diplococci. Injected three cultures "Smith"; no effect. This monkey showed remarkably little depression following the injections, and was soon in a normal condition.

It is somewhat remarkable that the later injections failed to influence the result of the first injection, which evidently reached the spinal canal. I have observed many other examples of the relative innocuousness of the diplococcus when introduced beneath the skin and into the muscles. The effects of large doses given in this way may be nil, or a slight elevation of temperature may appear and remain for a few hours, or the animal show for a brief period disinclination for food. The next experiment is introduced as an example of sudden death, after apparent recovery from an inoculation.

*Monkey No. 21. Macacus rhesus.*—July 1, 1905, 10 A. M. Two thirds culture "Smith" injected. No fluid obtained prior to injection. No symptoms developed, but the canal must have been entered as lumbar puncture July 2, 12 M., yielded a small quantity of turbid fluid containing leucocytes and very few degenerating, intracellular diplococci. No growth was obtained on sheep-serum agar. No symptoms of disease were noted in this animal. July 6, found dead. At the autopsy more clear fluid than is usual escaped from the dura surrounding the cord. The lower segment of the cord was congested. No exudate was visible and the ventricles were not dilated. The pial vessels of the medulla were injected. Smear preparations from the spinal cord and several parts of the brain showed neither diplococci nor polymorphonuclear cells. The sections of the tissue show no inflammation or leucocytic infiltration except superficially in the lumbar region in the posterior columns of the spinal cord at what was probably the point of entrance of the needle.

In contrast to the ease with which the acute inflammations can be excited is the difficulty which attends the production in monkeys of a subacute form of meningitis. The intraspinal injections pro-

duce either an acutely fatal meningitis, or an acute disease from which recovery takes place rapidly. The animals which survive inoculation are usually well of the disease in three or four days. In rare instances, the animal lingers a victim of the disease for a longer period than this to succumb in the end. Monkeys which survive the second day after inoculation tend rather to recover than to die. By following in inoculated monkeys the changes in the cerebro-spinal canal by means of lumbar puncture, the progress of the infection can be traced and the result often predicted. Disappearance of the diplococci early from the canal is a good sign; early emigration of leucocytes is also a good sign; early emigration, active phagocytosis and dissolution of the diplococci, both within and without leucocytes, are very favorable signs. Proceeding in this way, I have been able to keep a small number of monkeys in a state of subinfection with the diplococcus for a period of several days or weeks. The period of successive inoculation was determined by the puncture and by the physical condition of the monkey. A state of resistance tended to develop which necessitated the employment of increasing amounts of cultures to produce visible effects; and in the end the run-down animal succumbed to the dose administered. In these animals the exudates were thicker and firmer and covered the base and the convex surface of the brain; and the ventricles were sometimes widely dilated and contained turbid exudates. The first of these experiments was made with a South American species of which the next protocol gives an account.

*Monkey No. 27. South American; Genus Cebus.*—October 7, 1905, 11 A. M. Injected suspension of one agar culture "Whitaker," three days old. No symptoms developed. October 9, 11 A. M., l. p.: free flow of turbid fluid, of which 1 c.c. was collected. Injected one "Smith" culture eighteen hours old. 1 P. M., animal crouched in corner; head down and resting in hands; indisposed to move. 5:30 P. M., condition about the same; l. p.: a small quantity of fluid containing leucocytes, but no diplococci obtained. Culture negative. October 12, 3 P. M., l. p. yielded a small amount of turbid fluid containing vacuolated leucocytes and mononuclear cells; no diplococci. One half agar slant culture of "Smith" injected. 9:30 P. M., animal depressed. October 13, animal on perch. October 16, appeared to be well. Suspension of two old "Smith" cultures injected. October 17, no marked effects. L. p. gave a few drops of faintly turbid fluid showing leucocytes with intact nuclei, of which some contain degenerated diplococci. October 18, l. p.: faintly turbid fluid flowed freely from the needle. C. s.: leucocytes and

mononuclear cells; no cocci. A number of vacuolated (or fatty) large mononuclear cells present. Injected one twenty-four hour culture "Smith." No symptoms developed. October 20, 3 P. M., l. p. gave small amount of turbid fluid showing leucocytes but no cocci. Culture negative. Injected one culture "Smith" twenty-four hours old. No symptoms. October 23, l. p.: 0.5 c.c. turbid fluid obtained containing leucocytes and a few red corpuscles. October 28, l. p. gave clear fluid containing mononuclear cells. Injected two "Smith" cultures. October 30, l. p.: a drop of turbid fluid obtained showing leucocytes with fragmented nuclei but no cocci. Injected two "Smith" cultures. October 31, l. p. yielded a few drops of turbid fluid showing many leucocytes, and large mononuclear cells; no definite cocci; culture negative. During this treatment the monkey lost in weight. No further injection was given until November 15, at 12:30 P. M., at which time a heavy suspension of a "Smith" culture, eighteen hours old (representing four agar slant surfaces) was injected. Symptoms rapidly developed; at 2:30, animal sick; l. p. gave with difficulty a few drops of blood-tinted fluid containing many agglutinated diplococci and a small number of leucocytes, of which some had included diplococci. 4 P. M., l. p. gave, with difficulty, little fluid in which diplococci were numerous. October 16, 9 A. M., monkey died. Autopsy. No excess of fluid in the meninges of the spinal cord, and no exudate was visible. Turbid fluid, in excess of the normal, infiltrated the meninges from the medulla to the optic commissure. The anterior and lateral pial surfaces of the hemispheres showed smaller and larger ecchymoses. The ventricles contained small quantities of turbid fluid; they were not dilated. Cultures from different parts of the brain and cord remained sterile. The cover-glass preparations from different levels of the spinal cord, the base and convexity of the brain, choroid plexus and ventricles differed from one another only in minor details. Several striking conditions were shown by them to exist in the membranes. In the first place, no, or almost no, normal cocci remained, but only fragments of swollen and degenerated cocci which were entirely or nearly so within cells. The cells consisted of polymorphonuclear leucocytes which predominated in number and contained the far greater number of cocci; and of much larger cells of wholly different aspect. The latter were several times as large as the leucocytes, and occasional examples were of colossal size (equal to 20 to 40 leucocytes). The nucleus was single, as a rule, and oval or crescentic in form, and vesicular. The protoplasm was pale, and the membrane at the periphery stained deeply. The smaller of these cells contained at times, degenerated cocci; and all may have ingested a variable number of ordinary leucocytes, or mononuclear cells. They were most numerous in the smears prepared from the exudate of the meninges of the medulla. The sections of the central nervous system present quite a different appearance from the other cases described. Although the exudate is thicker than in the previous instances, the chief novelty lies in the great proliferation of the fixed cells of the pia-arachnoid. The multiplication has taken place chiefly along the surface of the brain and at the outer line of the membrane. The new cells are relatively large and vesicular, and their occurrence in double rows gives at times a somewhat glandular appearance to the membrane. Polymorphonuclear leucocytes are numerous and distributed irregularly among the other cells. The degree

of encephalitis is far less than in many of the experiments. The main arterial branches of the brain show a more or less marked infiltration of the intima with leucocytes, and leucocytic invasion of the sub-intimal and inner-muscular layers of the vessels.

This experiment brings out the remarkable capacity which can be developed in the monkey to dispose of cultures of the diplococcus injected into the spinal canal. It is shown by the experiments that the actively bactericidal cells and secretions of the canal are capable of destroying, in a few hours, prodigious numbers of the diplococcus, and that agglutination of the diplococcus can also take place in the canal. Finally, it is shown that the monkey can be killed by an overdose of culture which causes death by poisoning and not by infecting the animals directly; for the lethal effect occurred in this case, although the diplococci were themselves quickly killed. Increased power, therefore, to destroy the diplococcus is not associated necessarily with an equally increased power to resist the toxic effects of the intracellular poison. This fact must have an important bearing upon a specific therapy of diplococcus meningitis.

The experiment is important in respect to the histological alterations present in the meninges. In no previous experiment did the exudate contain so large a number of cells which differed from leucocytes. The large cells described here occur regularly in cases of human meningitis and have been frequently observed. Their occurrence in numbers in this monkey serves to knit even more closely the experimental and the human diseases. Flexner and Barker,<sup>11</sup> and Councilman, Mallory and Wright<sup>12</sup> describe and figure these large cells among the leucocytic infiltration, and the latter investigators have traced their origin to the cells of the connective tissue and the lymph spaces of the pia-arachnoid. The histological conditions present in the membranes are comparable with those observed in cases of meningitis of longer duration in man.

*Monkey No. 32. Macacus rhesus.* April 19, 1906, at 12 M., given one agar culture "548" in the third generation. 3 P. M., 1 p., no fluid obtained. April 20, 12 M., 1 p., small quantity of clear fluid containing few leucocytes and free cocci. 6 P. M., animal depressed. April 21, active. April 22, 12 M., injected

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<sup>11</sup> A contribution to our knowledge of Epidemic Cerebro-Spinal Meningitis. *Amer. Jour. of Med. Sciences* 1894, CVII, 155, 259.

<sup>12</sup> *Op. cit.*, p. 102.

one culture; no apparent effect. April 23, 4 P. M., 1. p., very little fluid obtained. April 25, 12 M., one agar culture "Gratz." Fluid obtained before injection which produced no visible effect. April 26, one agar culture "Gratz." No symptoms. May 1, 3 P. M., 1. p., yielded slow flow of faintly turbid fluid showing leucocytes and larger cells but no cocci. Injected turbid suspension (equal to four agar cultures). 6 P. M., animal sick. Died during the night, May 2. Autopsy: The spinal membranes contained an excess of fluid. On opening the dura mater a gelatinous exudate was seen to occupy the meshes of the pia. A similar exudate covered the base of the brain and surrounded the foramen of Magendie. The convex surface of the brain was greatly altered by a moderately firm and adherent exudate of white color which occupied the pia-arachnoid. It covered the surface of the convolutions and filled the sulci, thus giving a remarkably smooth appearance to the brain (Plate IV, Fig. 6). The ventricles were considerably dilated and contained an excess of turbid fluid. Smear preparations showed the chief accumulation of leucocytes to be at the base of the brain and the lower portion of the spinal cord. The lateral surfaces of the brain showed fewer and more were present in the choroid plexus than in the fluid of the ventricles. Diplococci were present everywhere; they were intracellular and often degenerated. The number varied: they were abundant at the base and less numerous at the convexity of the brain. The mucous membranes of the nose showed, among the columnar epithelium, a number of fragmented leucocytes carrying diplococci indistinguishable from those of the brain (Plate V, Fig. 7). Sections of the spinal cord and brain bear out the findings described. The exudate occurs throughout the membranes covering these structures. It is thicker over the posterior than over the anterior surface of the cord, and while it is abundant over the convolutions, it is especially thick in the sulci. Little or no fibrin occurs. The exudate consists chiefly of polymorphonuclear leucocytes among which there are a large number of moderately large mononuclear cells. The brain substance is remarkably free from infiltration, while the lumbar cord shows invasion, about its posterior surface, to a considerable depth. The dura of the cord at certain levels shows leucocytic infiltration. Diplococci which are very numerous in the exudate, are almost entirely within leucocytes. They have been carried by these cells into the substance of the spinal cord.

A striking feature of the sections is derived from the width of the ventricles. As a rule, these appear as slits in the sections; in this case they are wide cavities. Usually, the ependymal epithelium is regular and relatively high; in this case, it is often depressed or flattened, and a considerable flattening of the choroid plexus, toward the wall of the ventricle, is noticeable. A considerable degree of sub-epithelial cellular proliferation has taken place in the walls of the lateral and fourth ventricles. Leucocytes are moderately abundant in the ventricles.

The dura mater was removed with its attachments to the ethmoid bone, and longitudinal sections prepared. The leucocytes surround the olfactory bulbs in dense masses, and they push along clefts and spaces in the dense membrane, existing now as elongated columns and now in circumscribed, abscess-like masses.

This experiment is valuable in showing that a condition of sub-acute infection of the meninges can be procured in the monkey by

the use of extraordinary means, and when the condition is present and the thick exudate incidentally covers and occludes the foramen of Magendie, a degree of internal hydrocephalus develops. The experiment enforces a point already made. The histological study of the dura mater at the anterior extremity shows that leucocytes pass into its substance for a considerable distance beyond the proper confines of the cranial cavity. The cells could, indeed, easily carry diplococci with them, in which case an explanation for the occurrence of leucocytes carrying diplococci in the nasal mucosa might be readily found.

The series of experiments described suffices to establish the varieties of effects which are produced upon the central nervous system of lower monkeys by means of intra-spinal injections of cultures of *Diplococcus intracellularis*. Among the histological appearances mentioned, there was one monkey, No. 17, which deserves more particular attention. I described, in this case, a condition of acute endarteritis of the type frequently found in cases of human meningitis. It would appear that this pathological condition occurs in many forms of meningitis as is shown by the investigations of Hektoen,<sup>13</sup> Councilman, Mallory and Wright,<sup>14</sup> and others. The latter authors state, indeed, that it is less commonly found in the epidemic (*Diplococcus intracellularis*) form of meningitis than in other forms (pneumococcus, etc.) of the disease. They figure a slight degree of the condition in a goat in which they produced experimental meningitis by injecting the diplococcus. I have encountered in still another monkey inoculated with the diplococcus very extensive acute endarteritis. The case will be described in full in my article on a serum therapy for experimental meningitis. A spider monkey (*Atelas ater*) was inoculated on December 2, 1905, at 12 o'clock noon, with a slant agar culture of the diplococcus. At 3 P. M. two cubic centimeters of anti-goat (diplococcus) serum were injected into the spinal canal. Death occurred December 3, 11 A. M. The lesion mentioned affects arteries of all sizes in the brain. At the base, over the medulla, rarely does

<sup>13</sup> The Vascular Changes of Tuberculous Lepto-meningitis, especially the Tuberculous Endarteritis, *Jour. Exp. Med.*, 1896, I, 112.

<sup>14</sup> *Op. cit.*, p. 77.



an artery escape. The arteries of the convexity are affected less uniformly, and those of the spinal cord least of all. The systemic arteries do not show the lesion. The subendothelial infiltrations of mononuclear and polynuclear cells may or may not occlude the vessel entirely (Plate V, Fig. 8). Both large and small vessels may be partially or wholly filled up with cells which, as a rule, push the endothelium before them. The endothelium is preserved unless lost from mechanical causes in making the sections. An appearance has been obtained in some vessels of a mushroom-like overlying of the endothelium by the new cells which have accumulated in the adjacent part of the vessel. The muscular coat is usually free from infiltration, as are the veins. This monkey showed an unusual degree of hæmorrhage into the meninges, and definite cortical abscesses, of small size, extending into the brain from the cortical membranes, or developing about the intra-cortical blood-vessels. The number of diplococci in the inflammatory exudate was not large.

The pathological effects and appearances which have been described are not, probably, specific. The experiments are, therefore, valuable, not because they show that *Diplococcus intracellularis* alone of the pyogenic micrococci can produce acute meningitis in monkeys, but because they prove that the diplococcus is capable of setting up in these animals lesions of meningitis which bear a close resemblance to the lesions which the same micro-organism causes naturally in human subjects. The finer the details of correspondence are between the natural and experimental disease, the more valuable do the experimental results become, and the more convincing are they that *Diplococcus intracellularis* is really the cause of epidemic meningitis in man. I have not undertaken to study all the common pyogenic micrococci in the manner of this study of the diplococcus, but I have made, for obvious reasons, a single experiment with *Micrococcus catarrhalis*. The culture employed was kindly given me by Dr. Elser. It fulfilled the usual biological tests for that micro-organism.

*Monkey No. 28. South American; Genus Cebus.*—October 12, 1905, at 3 P. M., inoculated three agar slant cultures of *M. catarrhalis*. Clear fluid flowed from the needle before the injection. 9 A. M., animal sick; sat on

bottom of the cage. October 13, 9 A. M., condition unchanged. 3 P. M., very sick; head rested on hands. L. p., 9 A. M., yielded a very small quantity of fluid by using syringe. C. s., many leucocytes containing intracellular cocci in moderate numbers; a few cocci extra-cellular. The cocci appeared larger than the diplococcus; some stained feebly. October 16, animal had lost flesh but was recovering. Sat on perch. L. p., 3 P. M., with the syringe a few drops of faintly turbid fluid obtained. It showed small numbers of leucocytes with fragmented nuclei, but no cocci. This animal never fully recovered its flesh, and the hair entirely disappeared from the long tail. It was found dead on February 12, 1906. The autopsy showed the brain and cord to have returned, apparently, to the normal condition. Cover-slips were free of leucocytes and cocci; cultures remained sterile. An adequate cause of death was not found. Nearly all of this species of monkey contain adult filaria in the peritoneal cavity, and filarial embryos in the blood.

. . . . .

By means of the experiments recorded in this paper, it has been shown that the lower monkeys can be infected without great difficulty with *Diplococcus intracellularis* and made to reproduce the pathologic conditions present in man in cerebro-spinal meningitis. The experiments establish that the diplococci, when introduced in a low level of the spinal canal, distribute themselves in a few hours through the meninges and excite an acute inflammation, the exudate of which accumulates chiefly in the lower spinal meninges and the meninges of the base of the brain. The uniformity with which the chief exudate is found at the base of the brain and the rarity of its appearance in equally great amounts over the convexity is a fact of importance in the dynamics of the cerebro-spinal fluid since there is relation between the quantity of exudate and the accumulation of the diplococcus. This distribution of the exudate led me to doubt the validity of the reasoning which would ascribe this localization of the inflammation in man to the entrance into the meninges of the infective agent directly through the nasal membrane. This tendency to localization at the base of the brain in monkeys is especially interesting in view of the fact that it is usually only a short time before death that they lie down upon the side of the body. In comparing the experimental lesions in monkeys with the naturally developed lesions in man, note should be taken of the occurrence under both conditions of encephalitis and abscesses, of hæmorrhages, of proliferation of large cells of connective tissue and tissue spaces, of acute endarteritis, of inflammation of the dorsal

root ganglia, of internal hydrocephalus, of relatively small amount of fibrin in the exudates, of fibrinous and other thrombi, and of phagocytosis of diplococci and somatic cells.

The inflammation of the meninges extends in monkeys into the membranes covering the olfactory lobes and along the dura mater into the ethmoid plate and nasal mucosa. The nasal mucous membrane is found, in many instances, to be inflamed and beset with hæmorrhages. Smear preparations from the nasal mucous membrane, from the higher levels especially, have shown me polymorphonuclear leucocytes, often in numbers, carrying diplococci which presented the form, size, staining qualities and degenerations of the diplococci occurring in the same cases in the cerebral and spinal meninges. Thus far *Diplococcus intracellularis* has not been cultivated from the nose of the infected monkeys. I have, however, secured other Gram-negative diplococci with which, it may be stated, the intracellularis could not be confounded.

Although the pathological effects produced in monkeys are comparable with those occurring in the natural disease in man, there is no real correspondence in the relative degree of susceptibility of the two species. The quantity of an active culture required to cause marked symptoms or to bring about death from meningitis in monkeys, is prodigious if compared with the number of diplococci which probably suffice to produce infection in human beings. Moreover, the amount of multiplication of the diplococcus in monkeys, excepting possibly in the focal abscesses, is under the most favorable conditions, small; and I am not disinclined to believe that in many of the experiments no multiplication whatever took place.

The experiments, the details of which have been set down here, besides being an argument for the causative action of *Diplococcus intracellularis* in epidemic meningitis, form, also, the basis of an attempt to influence the progress and termination of the experimental infection in monkeys through the employment of anti-sera prepared from the diplococcus. The results of the experiments with the anti-sera are given in a separate article.

I wish to acknowledge the valuable aid rendered by my former assistant, Dr. H. S. Houghton, in the course of these experiments.

## DESCRIPTION OF PHOTOGRAPHS.

### PLATE II.

FIG. 1. Smear preparation from contents of brain abscess, monkey No. 1. Typical intracellular diplococci. Methylene-blue staining.  $\frac{1}{8}$  in. objective.

FIG. 2. Low magnification to show the relation of the exudation in the pia-arachnoid of the convex surface of the brain and the sulci, and the extension of the exudation along the cortical vessels into the brain.

### PLATE III.

FIG. 3. Spinal ganglion showing leucocytic invasion about and between the nerve-cells of the ganglion.

FIG. 4. Low magnification to show the degree of acute encephalitis, and the involvement of intracortical blood-vessels in the infectious processes.

### PLATE IV.

FIG. 5. Low magnification to show the exudation about and within the spinal nerves.

FIG. 6. Brain, natural size, from a case (Monkey No. 32) of subacute meningitis compared with the brain from a case of very acute experimental meningitis in which the congestion of the pial vessels is very marked.

### PLATE V.

FIG. 7. Smear preparation from inflamed nasal mucosa showing a leucocyte, partly broken, enclosing diplococci, lying among columnar ciliated epithelial cells.

FIG. 8. High magnification of an artery of the base of the brain showing the lesion of acute endarteritis.

I am indebted to the skill and kindness of Dr. Edward Leaming for the photographs illustrating this paper.

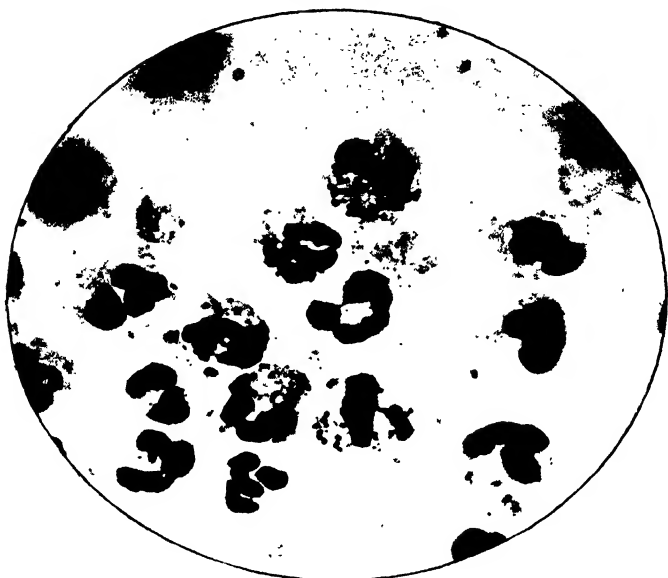


FIG. 1.



FIG. 2.



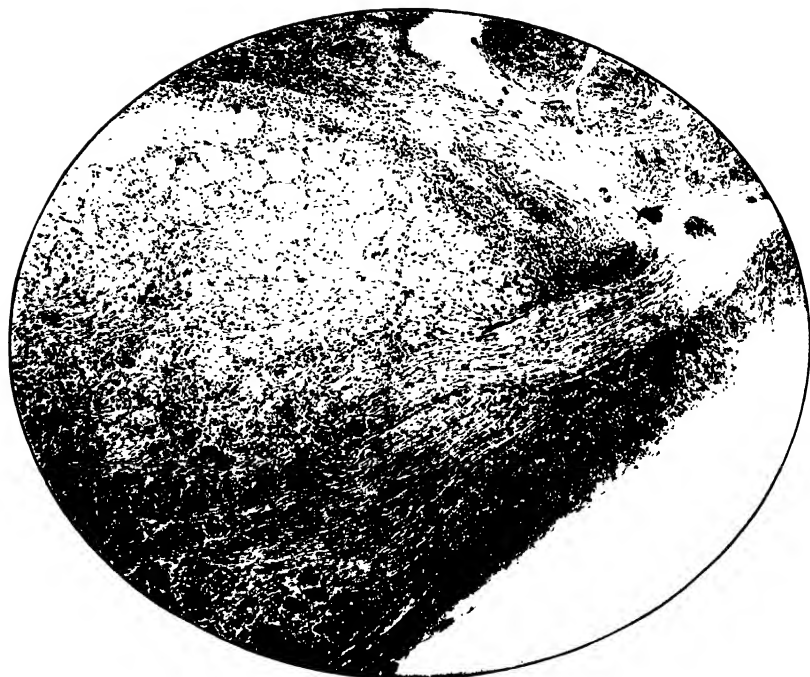


FIG. 3.

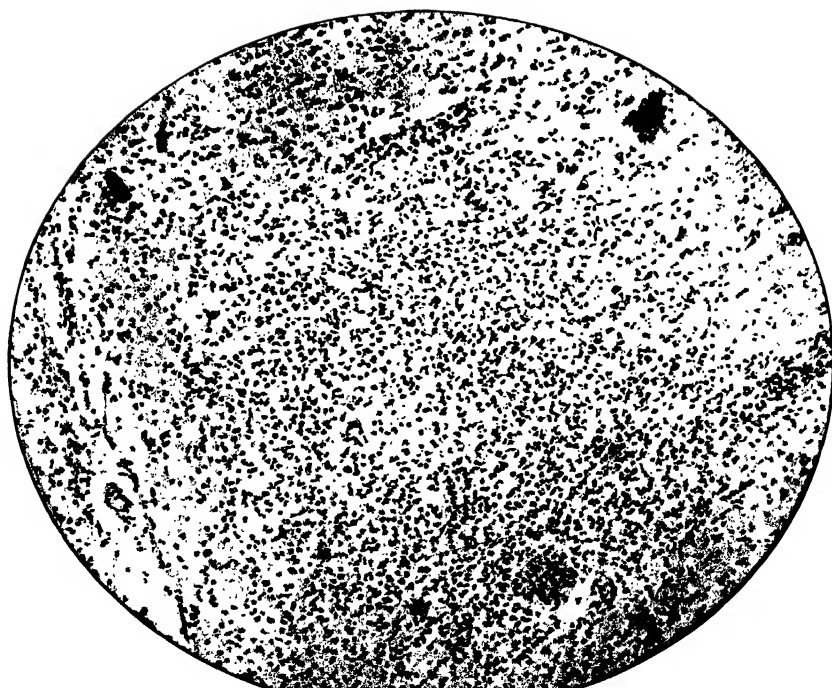








FIG. 5.







FIG. 7.

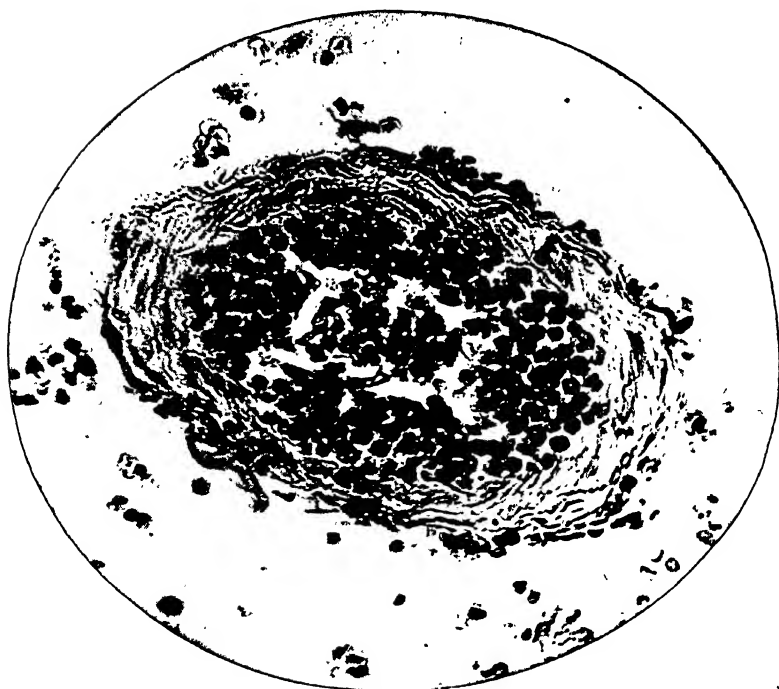


FIG. 8.



CONCERNING A SERUM-THERAPY FOR EXPERI-  
MENTAL INFECTION WITH DIPLOCOCCUS  
INTRACELLULARIS.

By SIMON FLEXNER, M.D.

## CONCERNING A SERUM-THERAPY FOR EXPERIMENTAL INFECTION WITH DIPLOCOCCUS INTRACELLULARIS.

By SIMON FLEXNER, M.D.

(From the Rockefeller Institute for Medical Research, New York.)

The high mortality of epidemic meningitis and the deplorable deformities caused by it demand that incessant effort be made to discover therapeutic measures which may mitigate the consequences of the disease. The epidemic through which the city of New York has recently passed, and the almost co-incident Silesian epidemic, have been scientifically fruitful in establishing more firmly the belief in the spread of the disease through immediate or mediate contact with the sick, and in tracing the common occurrence of *Diplococcus intracellularis* in the nasal and pharyngeal secretions of the sick, and the exceptional occurrence of the micro-organism in these secretions in the well who have been in contact with the sick. This mode of spread of the disease through directly and indirectly infected persons must come to exercise an important influence on the hygienic measures which will be enforced hereafter to limit the dissemination of the disease.<sup>1</sup>

<sup>1</sup> Goodwin and Sholly: The frequent occurrence of meningococci in the nasal cavities of meningitis patients and of those in direct contact with them. *Journal of Infectious Diseases*, 1906, Supplement No. 2, p. 21.

Flatten: Die übertragbare Genickstarre im Regierungsbezirk Oppeln, im Jahre 1905 und ihre Bekämpfung. *Klinisches Jahrbuch*, 1906, xv, 211.

Schneider: Idem im Regierungsbezirk Breslau, *ibid.*, p. 300.

Rieger: Idem im Kreise Brieg, *ibid.*, p. 321.

Schmidt: Idem im Regierungsbezirk Leignitz, *ibid.*, p. 341.

Flügge: Die im hygienischen Institut der königl. Universität Breslau während der Genickstarre-epidemie im Jahre 1905 ausgeführten Untersuchungen, *ibid.*, p. 353.

v. Lingelsheim: Die bacteriolog. Arbeiten der kgl. hyg. Station zu Beuthen, etc., *ibid.*, p. 373.

Göppert: Zur Kenntnis der Meningitis cerebro-spinalis-Epidemica mit besonderer Berücksichtigung des Kindersalters, *ibid.*, p. 313.

A less certain advance has been made in the therapeutics of epidemic meningitis. The one therapeutic measure growing out of the study of the epidemics in America and Germany which offers any hope is an antiserum for the diplococcus. It is true that the experience of the past is not favorable to the hope of achieving remarkable success by the employment of antibacterial immune sera. All indications point to the pathological effects of the diplococcus as being caused by endotoxic constituents; and thus far, according to many investigators, these endotoxins have failed to yield, by methods of immunization, active antisera which have proved valuable in the treatment of infectious diseases. Opinion is, however, considerably divided on this subject;<sup>2</sup> and in the absence of more certain methods of reaching the desired goal tests of antisera for the diplococcus are certainly justified. These tests can in the preliminary stages be carried out on certain animals, since the course of infection in them with the diplococcus is now fairly well understood.<sup>3</sup>

The main question which would seem to be involved in the search for an active antiserum against meningitis is whether the quantity of antibody which can be produced will suffice to neutralize such a quantity of the poison of the diplococcus as to influence the result of the infection. In fact, the problem may not be so simple, or, indeed, so hopeless, as this proposition indicates. It is, of course, important that neutralization of the poison should if possible be secured, but the effect of the restraint of growth and multiplication of the diplococcus may, at some periods of the disease, be of greater significance than the neutralization of free endotoxin. Fortunately, many agents, some of them quite indifferent, are able to affect the power of multiplication in the body of the diplococcus. It has been shown, indeed, that serum in the fresh state and after

Meyer: Bericht über rhinolog. Beobachtung bei der Genickstarre-epidemie, 1905, *ibid.*, p. 427.

Westenhoeffer: Pathologisch-anatomische Ergebnisse der oberschlesischen Genickstarre-epidemie von 1905, *ibid.*, p. 447.

Jehle: Entstehung der Genickstarre-epidemie, Wien. klin. Woch., 1905, xix, 25.

<sup>2</sup> Besredka, *Annales l'Institut Pasteur*, 1906, xx, 4.

<sup>3</sup> Attention is directed to the two previous papers of this series published in this number of the Journal.

heating to 60° C., preserves the power to destroy in test-tubes large numbers of the diplococcus, and sterile fluid inflammatory exudates possess this power in even greater degree. An antiserum, therefore, even though it contain relatively small amounts of antibodies, as indicated by neutralization experiments, may be effective beyond this calculated value by restraining the multiplication of the diplococci, possibly by reducing outright their number, and by supporting the power of resistance normally at the disposal of the body.

The conditions are made theoretically less discouraging, perhaps, because the main pathological lesions are limited to the cavity of the cerebro-spinal axis. They can, therefore, be brought directly under the influence of the antisera by injecting the latter into the spinal canal. A large advantage is gained by this circumstance. It is, on the other hand, discouraging to reflect that in monkeys infected with the diplococcus, severe cortical lesions already exist at the end of ten or twelve hours. The question arises whether these deeper lesions tend to appear as early in the human infections. In respect to this question it should be stated that observation is against the occurrence of any such development of the diplococcus in the early stages of the human disease as is represented by the prodigious number of diplococci required to be injected into monkeys to produce the rapidly lethal effect with which the cortical lesions are associated. It is worthy of note that the more slowly developed lesions in the monkey remain more superficial, agreeing in this respect with the more common lesions present in fatal cases of the human infection. Hence, some encouragement may be taken from the power of the antiserum to influence favorably the course of meningitis in the monkey, although it has been injected as late as six hours after the inoculation.

If we are at all permitted to apply test-tube experiments to what may happen in the body, it would not be remarkable if the normal serum of animals, and perhaps of human beings, proved to be beneficial to a degree when brought into direct relation with the focus of development of the diplococcus. At first sight, judging from test-tube experiments, it would appear as if the exudate, called out by the inflammation, should suffice to destroy the diplococci; this manifestly does not happen in many cases. Indeed, it



is found that incubation outside the body will even increase the number of diplococci in the inflammatory fluid withdrawn from the spinal canal. It is safe to assume, therefore, that the exudate withdrawn has been exhausted of its power to destroy the diplococcus. It is quite possible that the introduction of fresh serum, of the same species of animal, may be helpful by bringing quickly into contact with the diplococci a quantity of actively destructive serum. The results of some of my experiments show that normal serum reduces appreciably the toxic effect of given doses of the diplococcus.

In experiments upon the monkey there is a definite low limit, beyond which it is not safe to go, for injection of fluids into the spinal canal. The species which I studied contain a small amount only of free spinal fluid. If one attempts to inject several cubic centimeters of fluid, symptoms of pressure may develop. In this respect the monkey is far less satisfactory to treat by intraspinal injections than are human beings.

I am far from having any conviction that cerebro-spinal meningitis in man can be influenced favorably by injections of immune sera into the spinal canal, or elsewhere in the body. The experiments to be described merely show that guinea-pigs and monkeys, in which the conditions of infection can be controlled, can be saved from the otherwise fatal effects of the diplococcus by the use of antisera, and to a less extent by the use of normal sera and other fluids. A preliminary note on this subject has already been made.<sup>4</sup> The protocols show that the experiments on immunity were begun during the spring of 1905. While the work was in progress two papers on the same subject appeared in Germany.<sup>5</sup> The use of monkeys for testing the antisera by direct injections into the infected and inflamed cerebro-spinal canal has not been made by the other investigators whose experimental studies were confined to guinea-pigs. Jochmann injected an antiserum prepared in the horse into the spinal canal of several human subjects of epidemic meningitis. The number of cases was too few to permit any conclusion of the value of the injections; but they showed that the injection of

<sup>4</sup> *Jour. of Amer. Med. Assoc.*, 1906, xlvii, 560.

<sup>5</sup> Kolle and Wassermann, *Deutsch. Med. Wochenschrift*, 1906, xxxii, 16. Jochmann, *ibid.*, p. 20.

horse's serum into the inflamed canal is not attended with special danger.

My first experiments on guinea-pigs were made with goat's sera. A female goat had been injected twice with cultures from several sources (12) of the diplococcus within a period of two weeks. The injections were made subcutaneously and gave rise to tumefaction which soon disappeared. After the second injection the goat aborted. The first bleeding was made two weeks after the second injection. As the table shows the serum at this time had little or no immunizing power. The experiment was designed to test the effect of an injection (1) previous to the injection of the diplococci, and (2) after the inoculation of the diplococci. The serum in the first instance was injected at 5 P. M. the day before, and in the second instance two hours after the inoculation with the diplococcus. All the injections, except bouillon in one pig, were intra-peritoneal. The emulsion of the diplococcus was injected at 11 A. M., November 29, 1905.

Series No.	Weight in Grams	Protective Substance Injected.	Result.
144	189	"Immune" serum 0.03 c.c. 5 P.M. Nov. 28	Died 9 A.M. Nov. 30.
145	189	" " 0.04 c.c. " " "	Died 8 A.M. Dec. 2.
146	182	" " 0.05 c.c. " " "	Survived.
147	197	"Normal" " 0.05 c.c. " " "	Survived. Lost weight.
148	197	" " 0.10 c.c. " " "	" " "
149	220	Bouillon intraper. 1.0 c.c. " " "	" Weight 12/11 182 grams.
150	199	" subcut. 1.0 c.c. " " "	Died morning, Dec. 3.
151	190	"Immune" serum 1.0 c.c. 1 P.M. Nov. 29	Survived. Lost weight.
152	185	" " 2.0 c.c. " " "	" " "
153	192	"Normal" " 1.0 c.c. " " "	Died 5 P.M. Nov. 30.
154	190	" " 2.0 c.c. " " "	Died in night, Nov. 29.
155	190	Nothing: control	Died morning, Dec. 1.
156	210	Idem.	Died in night, Nov. 29.

The preceding experiment is of interest in showing the irregular action of the serum injections, and especially, as bringing out the fact that such an indifferent substance as bouillon can, if it is injected in advance of the diplococcus, impart power of successful resistance to the guinea-pig. It developed subsequently that the bouillon need not be injected into the peritoneal cavity to achieve this effect; one cubic centimeter injected subcutaneously in pigs of 225 to 250 gram weight, the day before inoculation, frequently

saves the animals. Using the goat serum of low protective value, the fact was determined that the protecting power of the serum has a definite limit (about 0.05 c.c. for pigs of 250 grams weight) under the most favorable conditions, namely, intraperitoneal injection the day before the infection. By using larger quantities (up to 1.0 c.c.) of serum simultaneously with the injection of the diplococcus the pigs can also be saved; subcutaneous injections of the serum (1.0 c.c.) in advance are effective. The smallest number of successful results is obtained in pigs in which the serum injections follow, after one or more hours, the infection. Several experiments were carried out in order to determine the fate of the diplococcus in the peritoneal cavity in the "protected" and "unprotected" animals. The plan was to withdraw fluid, after a suitable interval, by means of capillary tubes. The results were not wholly uniform, but in the main showed more rapid disappearance of the

Guinea-pigs weighing 230 to 260 grams, received 0.1 c.c. "immune" goat's serum intraperitoneally and subcutaneously, 1.0 c.c. bouillon intraperitoneally and subcutaneously, followed the next day by an emulsion of the diplococcus injected into the peritoneal cavity.

Series No.	How Injected.	Examination During Life.	Autopsy Findings in Peritoneum.
119	Goat's serum intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes containing a few diplococci; no extracellular diplococci.	Survived.
120	Goat's serum subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; very few leucocytes overlaid with diplococci.	Died during the night. 2 c.c. fluid exudate; more pus present than usual; many leucocytes including diplococci.
121	Bouillon intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Diplococci more numerous than in 120; no leucocytes.	Died during the night. 3 c.c. fluid exudate; no leucocytes; many diplococci.
122	Bouillon subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes; some intracellular diplococci; very few extracellular ones.	Survived.
125	Control; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; almost no leucocytes.	Died during the night. Usual p. m. appearances; very large number of diplococci free; almost no leucocytes. The omentum contains some leucocytes including cocci.

diplococcus from the peritoneum of the treated as compared with the untreated pigs. Exudation of cells was more abundant in the former animals.

The injections of the goat were carried on subsequently, during and after lactation, until the end of February, 1906. Blood was withdrawn from time to time and the serum tested for its protective and therapeutic value. The results were never uniform, but the general indication was that the protective properties were increased measurably. The animal fell ill on March 15, 1906, and was bled to death. The serum obtained from this bleeding was used for many subsequent experiments. It was found by simultaneous injection to protect small pigs (190 to 200 grams) against a twelve hour fatal dose of the diplococcus in quantities varying from 0.5 to 0.01 c.c., but not regularly. On the whole, as the next experiment given in detail will tend to show, the serum had acquired protective and therapeutic properties.

An emulsion of the diplococcus was prepared of which 0.5 c.c. caused death in the control pigs in about eighteen hours. Three series of pigs were tested; the first received the serum and emulsion simultaneously, the second the serum two hours, and the third the serum four hours after the emulsion. One tenth c.c. of serum was injected in each pig. The emulsion was given intraperitoneally.

Series No.	Serum Injected.	Result.
102	Intraperitoneal; simultaneously.	Survived.
103	Subcutaneous; "	Died in 4 hours.
105	Subcutaneous; after 2 hours.	" 16 "
106	Intraperitoneal; " 2 "	Survived.
107	Subcutaneous; " 4 "	Died in 56 hours.
108	Intraperitoneal; " 4 "	Survived.

I shall supplement this table by another in which the goat serum is compared with an immune serum made in monkeys (*vide infra*). This experiment has, in this place, a two-fold value in showing that certain of the series of tests proceed in a regular manner, and the two immune sera have about equal protective value for guinea-pigs. The emulsions of the diplococcus killed six control pigs in from nine to fifteen hours. The injections were simultaneous.

The next table given shows that the sera of the goat and the monkey were both capable of protecting guinea-pigs from fatal doses of the diplococcus by simultaneous injection, and the previous

Series No.	Weight in Grams.	Serum Injected.	Result.
106	190	0.1 c.c. goat, interperitoneal.	Survived.
107	188	Idem.	"
108	180	0.1 c.c. monkey, intraperitoneal.	"
109	185	Idem.	"
110	185	0.1 c.c. goat, subcutaneous.	"
112	175	Idem.	Died in 12 hours.
114	195	0.1 c.c. monkey, subcutaneous.	Survived.
117	178	Idem.	"
175	210	0.1 c.c. goat serum, intraperitoneal.	"
178	205	0.2 c.c. " " "	"
181	170	0.5 c.c. " " "	"
188	185	1.0 c.c. " " "	"
182	205	0.1 c.c. monkey serum, intraperitoneal.	"
183	182	0.2 c.c. " " "	"
186	175	0.5 c.c. " " "	"
187	172	1.0 c.c. " " "	Died in 30 hours.

one indicates that the infected pigs can with less uniformity be rescued at the expiration of two to four hours after inoculation. The striking results of the last table are, however, relative merely, since it is found that certain normal goat sera possess a power that it almost equal to that of the immune serum in protecting by simultaneous inoculation. On reweighing, four days later, the surviving pigs of the last tabulation a marked loss of weight was found to have taken place in those which received the sera subcutaneously and not in those receiving it into the peritoneum.

It would be extremely hazardous to express, on the basis of my experimental results, the relative value, as protective agents against the diplococcal infection in guinea-pigs, of normal and immune goat sera. I was restricted to the use of a female goat for the purpose of immunization, which was, unfortunately, during a part of the process, lactating. This fact could account for the low relative value of the serum. I did not, however, find the injection of normal goat serum following the inoculation of the diplococcus, so effective as the injection of the immune serum in this way. But here again, I was, I should state, limited in this study to three specimens of normal serum. On the whole, the immune serum saved more guinea-pigs than the normal serum. I am not, however, convinced that in the instances of preceding or simultaneous injection of the serum, in respect to the inoculation, any very great stress can be laid upon the inherent antitoxic, or even bactericidal power of the specific serum, since, in the former case, the exudate

caused to be poured in the peritoneal cavity has itself, in vitro, the power of suppressing large numbers of diplococci, and, in the latter, the sera possess this power only in less degree than the exudate. If, however, it can be shown that the immune sera are more effective as therapeutic agents than normal sera, and by simultaneous and preceding injections, also, then it will have to be conceded that they contain some useful elements absent from the normal serum or present in it in less amount.

In the course of these experiments the effort was made to produce an immune serum in the horse. For this purpose, recent cultures were injected by Dr. Jobling, first subcutaneously and later intravenously. The doses had to be carefully chosen on account of the high sensibility of the horse to the diplococcus. The subcutaneous injections produced local swellings which frequently softened and discharged externally. After a period, intravenous injections of autolysates were begun; but the alarming symptoms which followed almost immediately the injection of a few cubic centimeters of this fluid caused a return to the use of cultures and autolysates by the subcutaneous method. After about five months of intermittent injection the serum was collected and compared for protective value on small guinea-pigs. I employed as controls several samples of normal horse serum kindly supplied by Dr. Park of the Board of Health. I shall give a few typical experiments from which it would appear that on the whole the serum of the treated horse has greater protective value than the serum of normal horses.

Two strains of the diplococcus of moderate virulence were employed in the tests. The suspensions were made in the morning, control pigs being inoculated with them immediately. About four hours later, the rectal temperatures indicated the probably fatal doses. It was subsequently found that 0.1 c.c. of the suspensions caused death in less than twelve hours and represented, for susceptible pigs of about two hundred grams, two or three fatal doses. The inoculation of the pigs for the experiments on immunity was made in the afternoon of the same day. This procedure is rendered necessary by the rapid deterioration of the suspensions in salt solution and Ringer's fluid which sometimes takes place at

the refrigerator temperature. The deterioration is associated with loss of viability and not with striking morphological changes in the diplococci, from which fact I have been led to believe that multiplication for a period of the inoculated diplococci may be a necessary condition of the lethal dose.

Three control guinea-pigs were injected at 10 A. M. with 0.1, 0.2, and 0.3 c.c. of a suspension of "Mt. Sinai culture" of the diplococcus. The pig receiving the largest dose died at 4.30 P. M., at which time the temperatures of the other pigs were 34.6 and 34.8° C., respectively. A series of guinea-pigs which had been injected into the peritoneum at 4 o'clock the previous day with normal and immune horse serum, were now inoculated into the abdominal cavity with the suspension of the diplococcus. The two remaining control pigs died at 11.30 P. M. the same day. All the pigs weighed between 165 and 190 grams.

#### NORMAL HORSE SERUM.

Series Number	Quantity of Serum Injected 24 Hours Previously.	Quantity of Suspension Injected.	Result.
68	0.01 c.c.	0.2 c.c.	Died midnight.
69	0.02 "	" "	Died 3 A. M.
72	0.05 "	" "	Died midnight.
75	0.07 "	" "	Survived.
78	0.10 "	" "	Died midnight.
82	0.30 "	" "	Died 1 A. M.

#### IMMUNE HORSE SERUM.

47	0.01 c.c.	0.2 c.c.	Died 3 A. M.
50	0.02 "	" "	Survived.
58	0.05 "	" "	Died 9 A. M.
59	0.07 "	" "	Survived.
61	0.10 "	" "	Died 3 A. M.
66	0.30 "	" "	Survived.

This result, while it is not remarkably good, does, on the whole indicate that the immune serum possesses greater power of protection than the normal serum. The condition of the experiment was fairly severe, since the dose of suspension represented about five fatal doses; but what should be especially noted is the varying sensitiveness of the inoculated pigs owing to which the tests give quite irregular results.

The next tabulation, which would seem to bring out the same fact, gives the result in a series of simultaneous inoculations. The

mixtures of suspension and serum were permitted to stand at the warm room temperature for half an hour before injection.<sup>6</sup>

## NORMAL SERUM NO. 1.

Series Number.	Serum Injected.	Suspension Injected.	Result.
51	0.01 c.c.	0.1 c.c.	Died after 8 hours.
54	0.02 "	0.2 "	" " " "
46	0.10 "	0.5 "	Survived.

## NORMAL SERUM NO. 2.

Series Number.	Serum Injected.	Suspension Injected.	Result.
49	0.01 c.c.	0.1 c.c.	Survived.
43	0.02 "	0.2 "	"
38	0.10 "	0.5 "	Died after 8 hours.

## IMMUNE SERUM.

Series Number.	Serum Injected.	Suspension Injected.	Result.
41	0.01 c.c.	0.1 c.c.	Survived.
37	0.01 "	0.1 "	"
34	0.02 "	0.2 "	"
44	0.02 "	0.2 "	"
42	0.10 "	0.5 "	"
39	0.10 "	0.5 "	"

Two other kinds of antisera were prepared and tested upon guinea-pigs. The first was made by injecting large rabbits with the peritoneal exudate of guinea-pigs which succumbed to intra-peritoneal inoculation with the diplococcus. The exudates were toluolized, freed from cells, the toluol was removed by evaporation at a low temperature, and injected into rabbits. The antisera obtained from the rabbit exercised a degree of protection which can be expressed as follows: simultaneous injections of the diplococcus and the antiserum into the peritoneal cavity tend to give protection; separate simultaneous injections, diplococcus into the peritoneum and serum under the skin, are effective in proportion to the quantity injected. Small doses of the serum (0.1 c.c.) do not protect, but they delay the lethal effect; larger doses (0.5 c.c.) prevent the lethal effect. Dosage of rabbit serum proved to be important; too little failed to protect, and too much (0.5 c.c. into the peritoneal cavity) prejudices the result by reason of its own toxicity. The pigs which received the culture and this dose of serum died soon after the controls. The second was an homologous serum

<sup>6</sup> It is my intention to make a later report, when the immunization shall have gone farther, upon the effects of the serum of the immunized horse as regards its power of protection for guinea-pigs and for monkeys.



yielded by large guinea-pigs which were injected at intervals for several months with cultures of the diplococcus, the peritoneal exudate from other guinea-pigs, and the autolysate, which proved not to have greater protective value than normal guinea-pig's serum.

If the result of these attempts to produce an antiserum for *Diplococcus intracellularis*, which should be effective in the experimental infection with the diplococcus in the guinea-pig are reviewed, they cannot be held to be particularly promising. Under the severe conditions of the experiments, the most that can be said is that various agents—bouillon, normal sera, immune sera—can at times affect favorably the course of the experimental infection; the lead in respect to this influence being taken rather by the immune sera. It is to be recalled that in small guinea-pigs the experimental infection is rapidly fatal; that the prostration of the pigs develops very quickly, and the animals are often moribund in six to eight hours after inoculation. To influence very favorably and systematically a pathological process which progresses as rapidly as this, would be, perhaps, an achievement. As the experiments show, this can be done, although not wholly in this degree, by appropriate dosage of certain antisera.

The next experiments to be reported relate to monkeys inoculated with the diplococcus and treated with anti-diplococcus serum made in the monkey. Two large monkeys (*Macacus nemestrinus*) were immunized, for the production of an homologous serum, by injecting them subcutaneously with exudates from the peritoneal cavity of guinea-pigs succumbing to diplococcus infection, and with emulsions of the diplococcus. The injections were made at intervals for a period of nine months, after which the animals were bled to death, and the sera tested. Before giving the protocols of these experiments, the chief facts of a much earlier experiment to influence the course of meningitis in a monkey by means of the antiserum of the goat should be given.

December 2, 1905, each of two spider monkeys (*Ataies ater*) was inoculated with one agar slant culture of the diplococcus. Fluid flowed from the needles before injection. 3 P. M., control sick; mate to this perhaps not quite so sick. Into the spinal canal of the latter 2 c.c. of goat's immune serum were injected. The immediate effect was alarming: animal relaxed, heart's action tumultuous,

respiration sighing. The symptoms passed off in 10 to 15 minutes. L. p. before injection of serum showed in each many diplococci; no leucocytes. 9 P. M., control in stupor from which he could be aroused; easily handled. Responded to introduction of needle for l. p., no fluid obtained; point of needle carried a small amount of exudate which showed many leucocytes and a small number of intracellular oocci. Rectal temperature  $40^{\circ}$  C. Serum treated, less stupid than control; could be handled alone. No fluid obtained by l. p. The small amount of exudate on needle showed leucocytes some of which contained diplococci. Rectal temperature  $36^{\circ}$  C. December 3, 6 A. M., both still very sick; 8 A. M., serum treated animal very much depressed; 10 A. M., serum treated animal dead; control brighter. The latter animal finally recovered. Autopsy on serum treated monkey. The spinal canal showed hæmorrhagic imbibition of the membranes of the lower third of the cord. The pia-arachnoid of the cord was infiltrated with gelatinous-œdematous exudate. Small hæmorrhages beset the pia-arachnoid of the cortex, and the meninges of the brain were infiltrated with an exudate similar to that of the spinal cord. The ventricles contained turbid fluid in small amount. Microscopical examination of sections of the brain and cord show the exudate to be moderate in amount, and to be thicker over the convexity than over the base of the brain. The hæmorrhages in the membranes of the spinal cord are large, and of the brain small. The most striking lesion is an acute endarteritis which effects all sized branches of the arteries of the brain and cord. Very few diplococci can be found.

The symptoms in this animal and the lesions found at autopsy were taken to indicate that goat serum cannot be injected with impunity into the inflamed spinal canal of monkeys.

Preliminary to the tests of the antisera prepared in the two monkeys, the lethal dose of the diplococcus had to be established. A recently isolated culture (Mt. Sinai 596), which had proven virulent for guinea-pigs, was chosen. Two control monkeys were inoculated (1) with 0.5 c.c., (2) with 1.0 c.c. of a suspension of the culture. Brief histories follow:

Control No. 1. *Macacus rhesus*. June 27, 1906, 11 A. M., given 0.5 c.c. suspension. 6 P. M., very sick; June 28, 9 A. M., brighter; 12 M., l. p. small amount of thin fluid obtained; animal very weak. June 29, 8 A. M., moribund; chloroformed at 1 P. M. Autopsy: The membranes of the cord and brain were pale; little visible exudate. C. s. show leucocytes in small numbers over cord and brain; and a few intracellular diplococci. Sections of the tissues confirm these findings; the inflammatory exudate is small in quantity; no marked lesions of the nervous tissue itself are to be seen.

Control No. 2. *Macacus rhesus*. June 28, 12 M., 1.0 c.c. of same emulsion injected (in refrigerator over night). 6 P. M., monkey sick; 9 P. M., very sick; June 29, 9 A. M., died. Autopsy: The meninges were injected and contained small hæmorrhages. The fluid in the pia of the cord was increased, and turbid. The meninges of the brain and the ventricles contained similar fluid in excess; the basal meninges the largest quantity. C. s. show leucocytes and

diplococi throughout the membranes. The spinal membranes contain the largest number of free diplococci. Cultures positive. Sections of the tissues show the lesions of an acute inflammation of the meninges and of the superficial portion of the cortex of the brain.

In making the serum tests, the larger dose of emulsion was always employed. Brief protocols of the experiments follow:

Experiment 1, July 3, 1906: Medium sized *Macacus rhesus* given at 11 A. M. 1.0 c.c. emulsion of diplococcus "596" together with 1.0 c.c. of monkey anti-serum No. 1. Fluid flowed from the needle before injection. July 4, 9 A. M., monkey appeared normal; active; on perch. L. p. yielded several drops of faintly turbid fluid, which showed on cover-slips free cocci, and leucocytes, some of which contained diplococci in moderate numbers staining feebly. Cultures from the fluid negative. July 5, 11 A. M., monkey apparently well. Lumbar puncture gave a small amount of clear fluid, which contained neither leucocytes nor cocci. Cultures negative. January 10, 1907, the monkey remained well.

Experiment 2, July 5, 1906: Medium-sized *Macacus rhesus* given 1.0 c.c. suspension of culture "596" at 11:45 A. M. Fluid flowed from needle before injection. At 2 P. M., the monkey was sick. L. p. gave a small quantity of rather thick, opaque fluid. One cubic centimeter of immune serum (monkey No. 2) injected rapidly. Immediately at conclusion of injection pressure symptoms of an alarming character developed. The animal was prostrated for two hours, after which it slowly got better. At 6 P. M., it responded to disturbance. At 8 A. M., next day, the monkey was up and appeared well. It remained so subsequently (January 10, 1907). The cover-slips from the lumbar puncture showed many polymorphonuclear leucocytes and a small number of lymphocytes, many extracellular and a few intracellular diplococci.

Before the next experiment with the serum was made, the culture "596" was again tested for virulence.

A medium-sized *Macacus rhesus* was given 1.0 c.c. emulsion at 10:30 A. M., July 11. 10 P. M., animal sick; on bottom of cage, July 12, 9 A. M., l. p., small quantity of a thin white exudate obtained. C. s. many leucocytes and few diplococci. Died 12:30 P. M. Lived about thirty-eight hours. The lesions found at autopsy were characteristic; exudate existed over the cord and brain, the base of the latter being chiefly affected. The cortical vessels were injected. C. s. showed cocci in the inflammatory exudate of the cord and base of the brain, and fewer in the exudate of the convex meninges and the ventricles. Sections of the brain and cord show marked inflammatory lesions of the usual character. Diplococci are abundant in the exudate.

Experiment 3, July 13, 1906: Moderately large *Macacus rhesus* given at 11:15 A. M. 1.0 c.c. emulsion of Coccus "596." Although no fluid was obtained, the canal was certainly entered; 4 P. M., animal sick; 5 P. M., depressed, but still sat up. L. p. gave a small quantity of turbid fluid, but during the operation the animal collapsed; 1.0 c.c. of immune serum from monkey No. 2 injected slowly. The monkey was watched until 10 P. M.; no progress of the disease. July 14, 10 A. M., animal appeared well. No future symptoms developed up to January 10, 1907. The fluid obtained by l. p. showed many leucocytes with an occasional intracellular coccus, and extracellular cocci.

Experiment 4, July 27, 1906: At 8:45 A. M., a medium-sized *Macacus rhesus* was given one full agar slant culture "610," 18 hours old, suspended in salt solution, into spinal canal. Fluid obtained before injection. At 11 A. M., 5 c.c. of antiserum from monkey No. 2, were injected into the skin of the thigh. Before the serum injection the animal was sick; it lay half down on the bottom of the cage. No immediate effect followed from the injection, but the symptoms did not progress. The next day the animal appeared well. L. p. was unsuccessful after the injection until the next day, at which time clear fluid, containing neither cocci nor leucocytes, was obtained. The control for this monkey was a much smaller and weaker monkey of the same species, which succumbed in eight hours.

I do not regard this experiment as entirely free from doubt, but as I was unable to obtain more monkeys at this time, the experiment could not be repeated then.

The series of experiments was up to this point successful, and they indicated that an antiserum to the diplococcus could prevent the development of severe symptoms from following the injection of the cultures of the diplococcus into the spinal canal, and cause arrest of the symptoms which had already set in. This series of tests is, of course, incomplete without corresponding experiments with normal serum with which they may be compared. The latter will follow. But before citing them I wish to record a failure under conditions which, in view of the foregoing results, was wholly unexpected.

Experiment 5, July 20, 1906: Medium-sized *Macacus rhesus* given at 12 M. 1.0 c.c. of emulsion of Coccus "596"; 3 P. M. 1.0 c.c. of antiserum injected intraspinally. This monkey was sick when given the serum and the symptoms progressed fairly rapidly. At 11 P. M. the animal was much prostrated and sat in the cage with head depressed. It died about 7 A. M., July 21. The autopsy showed a very unusual amount of exudation in the membranes of the cord. Cover-slip preparations showed a purulent exudate with large numbers of diplococci, all within polymorphonuclear leucocytes. The exudate in the meninges of the brain and cord showed the diplococci in the same condition of complete phagocytosis, although the number of leucocytes and diplococci was smaller. The fluid withdrawn by lumbar puncture before the serum injection contained large numbers of diplococci and very few leucocytes; only an occasional leucocyte contained diplococci. Cultures made at the autopsy from the meninges of the cord, medulla and cortex, and from the lateral ventricle were positive; those from the heart and bone marrow of the femur were negative. Examination of sections of the brain and spinal cord bear out the macroscopic appearances. The exudate is remarkably thick over the entire nervous system, and is composed exclusively, or nearly so, of leucocytes. The lateral ventricle is shown to have been dilated and to contain pus cells. The brain tissue has escaped invasion with leucocytes, and the intracortical blood vessels are free from thrombi and do not show the perivascular infiltration with pus cells which is commonly present. The spinal cord at the level of the injection shows a superficial invasion with leuco-

cytes, but the higher levels do not show this condition. The dura is, in the former locality, infiltrated with pus cells.

The control for this experiment was a smaller *rhesus* monkey. The injection was made July 19, at 2 P. M., and as symptoms had failed to develop from the small dose given, a second injection of one third culture was made at 6 P. M. At 9 P. M., animal sick; lying down, but when disturbed rose and looked distraught. July 20, 7:30 A. M., lay on bottom of cage, but on being disturbed rose and displayed marked nystagmus; resumed recumbent position. Depression increased during the morning; 12 M., died. The autopsy showed a general thin exudate in the meninges, marked chiefly over the medulla. Cover-slips showed a remarkably large number of cocci which, while chiefly within leucocytes, were abundant outside. In no other experiment was so large a number of micro-organisms seen. A suspicious circumstance was found in the appearance of short chain-like groups of cocci, 4 to 6 members long. The cocci were Gram-negative and in size like the diplococcus. Attempt at cultivation failed.

In summarizing these experiments, it may be said that by the employment of an homologous anti-diplococcus serum several monkeys were saved from death due to experimental infection with *Diplococcus intracellularis*. The conditions of the experiments were such that the inoculated monkeys could, by simultaneous injection of serum and culture be prevented from developing severe symptoms, although the diplococci persisted for a period in the spinal canal, and by separate injection of the culture, and six hours later of the serum, the already severely ill monkey could, apparently, be saved from certain death. The experiment in which the serum was used successfully by subcutaneous injection cannot be interpreted without suitable repetitions.

The tests with normal monkey serum to serve as controls for the above experiments were made so as to bring out two sets of facts. In the first place, the value of simultaneous injection of normal monkey serum and a quantity of culture which would cause death in the control animal within twenty-four hours, was studied. And in the next place, the value of the normal serum was studied in monkeys in which the dose of the culture was on the border line—that is, of such a size that certain monkeys survived and others succumbed after a greater period than twenty-four hours. As regards the second series of tests, it may be said that it appeared as if the injection of a mixture of the normal serum and the culture led, in certain cases, to the survival of the monkey after a period of illness which was sometimes severe. The first symptoms ap-

peared very soon—within one or two hours—and grew in intensity for five or six hours, after which they receded. I am, therefore, inclined to attribute to normal serum employed in this way, a certain definite protective value.

The results in the first class of experiments were different. I found that the normal serum not only failed to save the inoculated monkeys, but the injection of the mixture of culture and serum might even hasten the fatal outcome. I wish to speak with some reservations on this point, for I was greatly hampered in this entire series of tests by great difficulty in obtaining at the time a suitable number of monkeys for the experiments. The study must, indeed, be carried much further before a final answer can be given as to the availability of a serum therapy for this experimental diplococcal infection in monkeys. There follow two brief protocols relating to the use of normal serum with a certainly fatal dose of the diplococcus. Two animals of the same species—*Macacus rhesus*—of equal size were employed.

*Control Monkey.* At 12 o'clock noon, one cubic centimeter of a suspension of the diplococcus fatal to guinea-pigs was injected into the spinal canal. Fluid flowed from the needle before injection. At 5 P. M., the monkey was sick; 9 P. M., lay on bottom of cage, but could be roused. Next morning comatose; died at 2 P. M. Survived the inoculation 26 hours. The lesions found at autopsy were characteristic. Cover-slips showed that the diplococci had to a large extent disappeared. The cultures were negative.

*Serumized Monkey.* At 12 o'clock noon, one cubic centimeter of the same emulsion used in the previous experiment (in refrigerator over night) mixed with one cubic centimeter of normal monkey serum and placed at 37° C. for half an hour, was injected into the spinal canal. Fluid was obtained before the injection. No immediate effects were noted following the injection. The monkey was already sick at 3:30 P. M., the symptoms increasing with great rapidity. Death took place at 8 A. M. the next morning. Survived 22 hours. The autopsy showed vivid injection of the meningeal vessels, and many small hæmorrhages over the cortical convolutions. Cover-slips showed a rich emigration of leucocytes and almost total disappearance of the diplococci. The cultures remained sterile.

I have no desire to attempt to apply, at this time, the results given here of the experiments with the various sera on guinea-pigs and monkeys to human beings the subjects of cerebro-spinal meningitis. The experimental results with the antisera were not sufficiently constant and striking to make this mode of treatment of human cases of cerebro-spinal meningitis of very hopeful au-

gury. On the other hand, it is not improbable that more active antisera, using appropriate means of immunization, may be produced. Possibly, such antisera may prove of value in the treatment by direct spinal inoculation, possibly even by intravenous or subcutaneous injection, in this hopeless disease. The evident disadvantages to which the human patient must always be subject, as compared with the animals used for experiments, arise from the difficulty often encountered of estimating exactly the duration of the disease, and applying the remedy at its most favorable stage. On the other hand, the exceptional cases only in man run so rapid a course, attended with such profound symptoms of intoxication, as are regularly seen in the inoculation disease in animals. The slower and more measured progression of the infection in human beings may, indeed, be a favorable circumstance, provided the treatment can be applied in the early stages and before too severe structural changes have taken place in the nervous system. The fact that normal serum exercises a certain degree of protection might possibly be taken advantage of in cases of human infection. It would, of course, be practicable to obtain normal human serum for such injections. This subject is one which, in view of the gravity of cerebro-spinal meningitis in man and the absence of any efficient therapeutic measure against it, would seem to deserve consideration.





## LEUCOPROTEASE AND ANTI-LEUCOPROTEASE OF MAMMALS AND OF BIRDS.

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Studies of Fr. Müller<sup>1</sup> and subsequent observers have shown that the polynuclear leucocytes of man and other mammals contain an enzyme which digests proteid and is particularly active in the presence of a weak alkaline reaction. One of us<sup>2</sup> has shown that a suspension of cells from an inflammatory exudate causes proteolysis both in an alkaline and in an acid medium, though digestion is more active in the former. It has further been possible to obtain from such cells a dry powder<sup>3</sup> which digests only in the presence of an alkaline reaction; this enzyme has been designated leucoprotease.

Proteolysis caused by cells of an inflammatory exudate in the presence of acid is more active in proportion to the number of large mononuclear phagocytes or macrophages which are present, while an emulsion made from the lymphatic glands which are situated near the seat of inflammation and contain such cells in immense number causes proteolysis in an acid medium and fails to digest in a neutral or alkaline solution; for convenience, this enzyme has been designated lympho-protease. Hence, each of the two types of phagocytic cells which are capable of ingesting and dissolving within their substance micro-organisms and other proteid-containing bodies is characterized by an enzyme, namely, the polynuclear leucocyte contains leucoprotease, which digests in a neutral or alkaline medium while the large mononuclear phagocyte, which is most abundant in the inflammatory exudate during the later stages of inflammation and often attacks and digests the smaller polynuclear cell, contains lymphoprotease which digests only in the presence of acid.

<sup>1</sup> Kossel, *Zeit. f. klin. Med.*, 1888, xiii, 149.

<sup>2</sup> *Jour. of Exper. Med.*, 1905, vii, 316.

<sup>3</sup> *Ibid.*, 1906, viii, 410.

Leucoprotease, like trypsin, digests in the presence of an alkaline reaction, while lympho-protease, like pepsin, requires an acid medium. Lymphoprotease is active in the presence of very weak hydrochloric acid (1/100 N.) but fails to act in the presence of that concentration of hydrochloric acid which is most favorable to the action of pepsin.<sup>4</sup> Leucoprotease, moreover, has been found to be far less active than trypsin. Further knowledge concerning the relation of the enzymes of leucocytes to the enzymes of the digestive tract is wanting.

The studies of Salkowski<sup>5</sup> have shown that organs kept at body temperature under conditions which prevent bacterial growth undergo self-digestion. Bondi<sup>6</sup> found that self-digestion of liver tissue is more active in an acid than in an alkaline medium. Hedin and Rowland<sup>7</sup> have shown that juice expressed from the spleen of the beef, horse, pig and sheep undergo especially active autolysis in the presence of an acid reaction. The reaction of the expressed juice is acid, but the addition of 0.1 per cent. hydrochloric acid increases proteolysis. In the presence of an alkaline reaction produced by adding from 0.2 to 0.37 per cent. of sodium bicarbonate, the degree of autolysis is diminished, but is, nevertheless, considerable. Subsequent observations<sup>8</sup> have shown that lymphatic glands, the kidneys and the liver contain proteolytic enzymes which are more efficient in an acid than in an alkaline medium. The juice expressed from voluntary muscle undergoes only slight autolysis which is not increased either by addition of acid or of alkali. The heart muscle contains an enzyme which resembles that of other organs and is more active in the presence of acid. Levene and Stookey<sup>9</sup> found that autolysis of nerve tissue and of testis is increased by acid.

Hedin<sup>10</sup> succeeded in separating from the spleen two enzymes, one of which, designated by him lieno- $\alpha$ -protease, acted in alkaline

<sup>4</sup> *Ibid.*, 1906, viii, 418.

<sup>5</sup> *Zeit. f. klin. Med.*, 1890, Suppl. zum xvii, 77.

<sup>6</sup> *Virchow's Archiv.* 1896, cxliv, 373.

<sup>7</sup> *Zeit. f. physiol. Chem.*, 1901, xxxii, 341.

<sup>8</sup> *Ibid.*, 1901, xxxii, 531.

<sup>9</sup> *Jour. Med. Research*, 1903, x, 212.

<sup>10</sup> *Jour. of Physiol.*, 1904, xxx, 155.

medium, while a second enzyme, which he called lieno- $\beta$ -protease, acted in the presence of acid. After spleen pulp had been digested in the presence of 0.1–0.2 per cent. acetic acid, that enzyme which digested in the presence of acid was found in solution, while from the residue, after extraction with three per cent. sodium chloride, precipitation with acetic acid and subsequent neutralization, an enzyme was obtained which acted rather strongly in an alkaline medium and much less in an acid. Hedin showed that the enzymes which he isolated not only caused autolysis, by digesting the substance of the cells which contained them, but were capable of breaking down other proteids, such as fibrin, casein and coagulated blood serum.

The cells of an inflammatory exudate obtained by injecting aleuronat into the pleural cavity of the dog, unlike all of the organs which have been mentioned cause more active proteolysis in an alkaline than in an acid medium. Another tissue has been found by one of us<sup>11</sup> to share this property, namely, the bone-marrow. A suspension of cells from the spleen, lymphatic glands, liver or kidneys caused much more active proteolysis of heated serum with acid than with alkali, while similarly prepared suspensions of cells from the bone-marrow were far more active in an alkaline medium. It is not improbable that the enzyme which is present in large amount in the cells of the exudate rich in polynuclear leucocytes and in the tissue from which the polynuclear leucocytes arise is identical with the similar enzyme which Hedin obtained from the spleen.

*The Relation of Anti-leucoprotease to the Globulins and Albumin of the Blood Serum.*—Hahn<sup>12</sup> first showed that normal blood serum has the power of inhibiting or wholly preventing the action of trypsin. According to Landsteiner<sup>13</sup> this anti-enzymotic action is not possessed by the serum globulin, precipitated by half saturation with ammonium sulphate, but is present in the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Glaessner<sup>14</sup> failed to confirm this observation, main-

<sup>11</sup> *Jour. of Exper. Med.*, 1905, vii, 759.

<sup>12</sup> *Berliner klin. Woch.*, 1897, xxxiv, 499.

<sup>13</sup> *Cent. f. Bakt.*, 1900, xxvii, Abt. i, 357.

<sup>14</sup> *Hofmeister's Beiträge*, 1904, iv, 79.

taining that the euglobulin fraction precipitated by one third saturation with ammonium sulphate inhibited the action of trypsin on coagulated proteid contained in Mett's tubes, while the pseudo-globulin subsequently precipitated by half saturation exhibited little, and the albumin fraction, no anti-enzymotic action. The results obtained by Landsteiner have been confirmed by Cathcart,<sup>15</sup> who found anti-tryptic action with the albumin, but not with the globulin fraction. This anti-enzymotic action of the serum is destroyed by a temperature of 70° C. but that of the isolated albumin fraction is destroyed by 55° C.

That enzyme of the spleen which acts in an alkaline medium and has been designated by Hedin lieno- $\alpha$ -protease is inhibited by the blood serum. Hedin found that the substance in the serum of the ox which checked this enzyme was contained in the albumin and pseudo-globulin fractions, whereas, in one experiment the euglobulin fraction slightly increased, in another slightly diminished, its activity. That part of the euglobulin fraction which was precipitated by dialysis of the serum was found to increase rather than diminish proteolysis caused by the splenic enzyme.

It has been shown by one of us<sup>16</sup> that proteolysis in an alkaline or approximately neutral medium caused by a suspension of cells from an inflammatory exudate is prevented by small quantities of the serum of the exudate or of the serum of the blood. This anti-enzymotic action is destroyed by a temperature of 75° C. but is unaffected by heating to 70° C. during an half hour. Baer and Loeb<sup>17</sup> observed that the serum of the blood exerted a similar action upon the autolytic enzyme contained in the liver, but since, as they believed, this property was little, if at all, altered by heat, even at the temperature of boiling, they did not think it attributable to a true anti-enzyme. The same inhibiting action was exerted by the albumin of the serum but was wholly lacking in the globulin.

The purpose of the following experiments has been to determine primarily if the power to resist the action of the leucoprotease of the polynuclear leucocytes is common to all proteids of the blood

<sup>15</sup> *Jour. of Physiol.*, 1904, xxxi, 497.

<sup>16</sup> *Jour. of Exper. Med.*, 1905, vii, 316.

<sup>17</sup> *Arch. f. exper. Path. u. Phar.*, 1905, liii, 1.

serum or if this anti-body is localized in a particular fraction of the serum. The somewhat discordant results obtained with antibodies for other proteolytic enzymes have been cited for comparison.

To separate the globulins and albumin of the blood, the method of fractioning the serum employed by Freund and Joachim<sup>18</sup> has been used. The blood serum after centrifugalization was diluted with three times its volume of distilled water. A small amount of precipitate, euglobulin, was obtained by adding a saturated solution of ammonium sulphate in quantity to cause one third saturation. The precipitate was washed, dissolved in distilled water, and dialyzed; it was again precipitated by one third saturation with ammonium sulphate and again dialyzed. To the filtrate obtained after one third saturation was added a saturated solution of ammonium sulphate in quantity sufficient to cause one half saturation. The bulky precipitate thus obtained was dissolved in water, dialyzed and reprecipitated. The filtrate was completely saturated with ammonium sulphate by the addition of dry salt. A bulky precipitate, albumin, was obtained and further purified as before. The fractions of serum obtained by this method were dissolved in a volume of water approximately equal to or in some instances double that of the serum employed.

Dried and powdered leucocytes prepared by the method previously described (leucoprotease), in weighed quantities, were allowed to act at 37° C. during five days on a measured quantity of coagulated proteid (five cubic centimeters of heated serum) in the presence of the various fractions which had been isolated. Digestion took place in closely stoppered flasks; the volume of each mixture was brought to twenty-five cubic centimeters by addition of 0.85 per cent. salt solution, and one cubic centimeter of toluol was added. The amount of nitrogen in substances incoagulable by heat has been measured by the Kjeldahl method, and for the sake of comparison is given in terms of cubic centimeters of 1/10 N. sulphuric acid. Since previous experiments had shown that the anti-enzymotic activity of the serum was slightly greater when the alkalinity of the medium was increased, to each mixture in the following experiment was added 0.2 per cent. of sodium carbonate;

<sup>18</sup> *Zeit. f. physiol. Chem.*, 1903, xxxvi, 407.

in subsequent experiments this addition was not made. Former experiments having shown that the anti-enzymotic action of the serum is destroyed by heat, the effect of the unheated fraction was compared with that of the same fraction previously heated to 75° C. during one half hour.

20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin	20.5 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin heated	16.1 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin	27.2 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin heated	21.05 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin	13.8 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin heated	29.7 c.c.

In order to test the action of the various fractions in the absence of leucoprotease, quantities of pseudo-globulin and of albumin equal to those previously employed were allowed to act on heated serum under conditions similar to those just described, addition of alkali being omitted. The control represents the amount of nitrogen in uncoagulable form contained in a given mixture before digestion.

10 c.c. pseudo-globulin + coagulated proteid	21.1 c.c.
Control	6.95 c.c.
10 c.c. albumin + coagulated proteid	10.8 c.c.
Control	9.6 c.c.

The experiment demonstrates that digestion is increased when the euglobulin and pseudo-globulin fractions are added to a mixture of leucoprotease and heated serum. Digestion is inhibited on the contrary by the albumin fraction. The second half of the experiment shows that the pseudo-globulin fraction contains an active proteolytic enzyme, while the albumin fraction fails to cause noteworthy digestion.

The following experiments confirm the results of that just described. The conditions of the experiments are the same, save that euglobulin and pseudo-globulin were not separated but were precipitated together by half saturation with ammonium sulphate. The globulin fraction was dissolved in a volume of water approximately equal to that of the serum from which it was obtained. The albumin fraction was dissolved in twice its volume of water.

20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin	33.45 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin heated	24.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin	7.5 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin heated	18.1 c.c.

The action of the two fractions on coagulated proteid in the absence of leucoprotease was further tested.

Coagulated proteid + 15 c.c. globulin	28.1 c.c.
Control	10.3 c.c.
Coagulated proteid + 15 c.c. albumin	7.05 c.c.
Control	6.55 c.c.

The globulin and albumin fractions used in the next experiment were dissolved in volumes of water approximately equal to that of the serum from which they were obtained.

20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin	25.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin heated	23.85 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin	13.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin heated	22.6 c.c.

The same quantities of the globulin and of the albumin were allowed to act upon coagulated proteid at 37° C. during five days in the presence of an approximately neutral, alkaline, and acid reaction:

Coagulated proteid + 5 c.c. globulin	16.3 c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. sodium carbonate	6.5* c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. acetic acid	3.7 c.c.
Control	2.3 c.c.
Coagulated proteid + 5 c.c. albumin	2.4 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. sodium carbonate	2.55 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. acetic acid	2.15 c.c.
Control	2.4 c.c.

\* Since this figure differed markedly from that obtained when the reaction of the medium remained approximately neutral (16.3 c.c.) it was suspected that an error had occurred in making the Kjeldahl determination; repetition of the test gave the figure, 13.2 c.c.

These experiments which show that the anti-enzyme for leucoprotease is present in the albumin of the serum and absent in the globulins are analogous to those of Landsteiner and of Cathcart who found anti-trypsin only in the albumin fraction of the serum. Hedin, it has been mentioned, found an anti-body for his lieno- $\alpha$ -protease in the albumin fraction, but present also, according to his observations, in the pseudo-globulin.

The globulin of the serum not only possesses no anti-leucoprotease but contains a proteolytic enzyme which is active under conditions similar to those which favor the action of leucoprotease. Delezenne and Pozerski<sup>19</sup> had observed that serum treated with chloroform

<sup>19</sup> *Compt. rend. Soc. de Biol.*, 1903, lv, 327, 690, 693.

digests proteid, and believed that the serum contained an anti-body which normally held this enzyme in check. Hedin<sup>20</sup> showed that a weak proteolytic enzyme which digests in an alkaline medium is present in the globulin of the serum and is inhibited by an anti-body which is mainly contained in the albumin. It is not improbable that this enzyme is identical with that which is present in the polynuclear leucocytes and in the bone marrow and with the similar enzyme of the spleen. In the blood serum this enzyme is held in check by the anti-body which is precipitated with the albumin; this anti-body which in given quantity doubtless holds in check only a limited quantity of enzyme,<sup>21</sup> is present in the serum in excess, so that the whole serum is capable of further anti-enzymotic action.

*Action of Anti-leucoprotease of Different Mammalian Species upon Leucoprotease of the Same and of Different Species.*—By study of the anti-enzymotic action of sera from animals of various species upon leucoprotease derived from different animals, it was hoped that evidence concerning the identity or multiplicity of such enzymes might be obtained; for if each of two enzymes bears a specific relationship to its own serum, it is improbable that the two enzymes are identical. Glaessner believed that the anti-tryptic action of the blood serum bears a specific relation to trypsin from the same species; he found that trypsin from the ox was more strongly inhibited by ox serum and trypsin from the pig by pig's serum than by other sera. The experiments of Cathcart<sup>22</sup> did not confirm this view, but were not decisive.

In order to test the inhibiting action of sera obtained from a variety of species upon the leucoprotease of the dog, a measured quantity (twenty milligrams) of the dry powder prepared from leucocytes, obtained either by injecting aleuronat into the pleural cavity or turpentine into the subcutaneous tissue, was allowed to act during five days at 37° C. upon a measured quantity of coagulated proteid in the presence of various sera. In the following experiment three cubic centimeters of the serum of dog, man, and ox were employed. To determine for comparison the proteolytic activity of

<sup>20</sup> *Jour. of Physiol.*, 1904, xxx, 195.

<sup>21</sup> *Jour. of Exper. Med.*, 1906, viii, 538.

<sup>22</sup> *Loc. cit.*



the enzyme, twenty milligrams were allowed to act upon coagulated proteid in the presence of three cubic centimeters of the various sera previously heated to 75° C. during one half hour, in order to destroy their anti-enzymotic action. The control represented the amount of nitrogen in uncoagulable substances present in the mixtures before digestion.

	Human serum.	Dog's serum.	Ox's serum.
Control	3.35 c.c.	4.65 c.c.	3 c.c.
With 3 c.c. heated serum	25.25 c.c.	26.5 c.c.	21.8 c.c.
With 3 c.c. unheated serum	7.4 c.c.	8.7 c.c.	7.6 c.c.

The sera of man and of ox act like that of the dog, and hinder digestion caused by the dog's enzyme. It is not improbable that a maximum degree of inhibition has been caused by these sera when three cubic centimeters of each were employed. In order to test more accurately the relative activity of the different sera, smaller quantities were employed; 0.5 and 0.25 cubic centimeters of the serum of dog, cat, goat, and pigeon were added to mixtures containing twenty milligrams of leucoprotease and a measured quantity of coagulated proteid.

	Dog's serum.	Cat's serum.	Goat's serum.	Pigeon's serum.
With 0.5 c.c. serum	9.9 c.c.	4.3 c.c.	4.75 c.c.	16.8 c.c.
With 0.25 c.c. serum	10.85 c.c.	4.25 c.c.	6.4 c.c.	17.6 c.c.

The proteolysis caused by twenty milligrams of the enzyme acting on the quantity of coagulated proteid used in the above mixtures is represented by 18.2 c.c., the control being 3.0 c.c.

The serum of the cat causes more complete inhibition of the dog's enzyme than the serum of the goat, but both sera are more actively anti-enzymotic than the dog's own serum. Digestion in the presence of equal quantities of pigeon's serum is little less than that caused by the unrestrained enzyme. In the following experiment the sera of dog and rabbit were compared, the effect of increasing quantities of these sera on twenty milligrams of dog's leucoprotease being tested. Since the coagulated proteid used for digestion (five cubic centimeters of heated dog's serum) was not the same in the two series, the results are not accurately comparable, but demonstrate that the inhibiting power of the rabbit's serum is considerably greater than that for the dog.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	22.15 c.c.	7.35 c.c.
With 0.5 c.c. serum	18.8 c.c.	5.35 c.c.
With 1 c.c. serum	10.6 c.c.	4.3 c.c.
With 2.5 c.c. serum	7.55 c.c.	4.9* c.c.
Without the addition of serum	24.2 c.c.	21.45 c.c.
Control	2.85 c.c.	2.85 c.c.

\* This figure is larger than that obtained when a smaller quantity of rabbit's serum was used because the mixture before digestion contained more nitrogen in uncoagulable form, 2.5 c.c. of dog's serum being represented by 1.45 c.c. and 2.5 c.c. rabbit's serum by 1.75 c.c.

In the second experiment the figures obtained are comparable, the same coagulated proteid being used for digestion in all of the tests.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	12.5 c.c.	6.45 c.c.
With 1.0 c.c. serum	6.5 c.c.	5.15 c.c.

Digestion caused by the enzyme used in this experiment, in the absence of serum, is represented by 20.05 c.c., the control being 4.05 c.c.

Since the enzyme of the dog's leucocytes is more markedly inhibited by the serum of man, ox, cat, goat, and rabbit than by the dog's own serum, it is necessary to determine if the corresponding enzyme of other animals bears the same relation to foreign mammalian sera. For this purpose, the attempt was made to obtain polynuclear leucocytes of the rabbit in quantities sufficient for the tests required. By injection of aleuronat into the pleural cavity of the rabbit, only a small quantity of exudate was obtained, and this exudate was so poor in cells that their suspension in salt solution completely failed to digest coagulated proteid. Injections of turpentine into the subcutaneous tissue of the rabbit caused exudation of serum and accumulation of dry friable material of opaque white color forming a layer adherent to the necrotic tissues which had been in contact with the injected turpentine. This material consisted in great part of polynuclear leucocytes held together by fibrin; typical suppuration with softening and solution of tissue was entirely lacking. The exudate was scraped from the surface of the exposed tissues and dried by the method previously described. The dry powder thus obtained was found to have weak proteolytic action when allowed to act at body temperature on heated serum. The anti-

enzymotic action of rabbit's, dog's and hen's serum was tested with this enzyme.

20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	3.7 c.c.
20 mgr. leucoprotease + coagulated proteid	4.15 c.c.
Control	2.5 c.c.

Since the amount of digestion in the experiment, even in the absence of serum, was insignificant, a larger quantity of the ferment was employed.

50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.45 c.c.
50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	4.05 c.c.
50 mgr. leucoprotease + coagulated proteid	7.6 c.c.
Control	2.45 c.c.

These two experiments have shown that leucoprotease derived from the rabbit bears to the two sera with which it has been tested a relation which is identical with that of dog's leucoprotease to the same sera; the enzyme from both animals is inhibited in greater degree by the serum of the rabbit than by that of the dog. Since the anti-body of one species has no specific relation to the leucoprotease of the same species, the experiments tend to support the belief that the leucoprotease of different mammalian species is identical.

The enzyme from the rabbit is much weaker than the similarly prepared enzyme from the dog; the anti-enzyme of the rabbit's serum is, on the contrary, much more active. Since experiments previously described have shown that suppuration with solution of fibrin and of necrotic tissue in the dog is associated with loss of anti-enzyme in the purulent exudate, it is not improbable that the well-known absence of typical suppuration with liquefaction of tissue in the rabbit is due to the weakness of the enzyme present in the polynuclear leucocytes and to the strength of the anti-body which opposes it.

*Anti-leucoprotease in the Serum of Birds.*—An experiment in which the anti-enzymotic power of pigeon's serum was compared with that of dog's, cat's, and goat's serum showed that the serum of the pigeon failed to prevent active proteolysis caused by dog's leucoprotease. The inhibiting action of hen's serum was further

tested with leucoprotease of dog and was compared with that of dog's serum. The following experiment shows the effect of 0.5 and of 1 cubic centimeter of these sera on twenty milligrams of dog's leucoprotease when allowed to digest coagulated serum under the conditions already described:

	Dog's serum.	Hen's serum.
With 0.5 c.c. serum	9.5 c.c.	14.65 c.c.
With 1 c.c. serum	6.05 c.c.	13.75 c.c.

Twenty milligrams of enzyme in the absence of serum caused digestion of coagulated proteid represented by 16.8 c.c. of 1/10 N. sulphuric acid, the control being 2.1 c.c.

It is evident that the anti-enzymotic action of the serum of both pigeon and of hen for leucoprotease of dog, though appreciable, is slight. Since sera of these birds differ from the mammalian sera examined, it has suggested itself that the enzymes present in the leucocytes of the bird might have peculiarities corresponding to this difference. To obtain inflammatory exudates containing leucocytes, aleuronat was injected into the peritoneal cavity of the hen, but from the resulting exudate a sufficient quantity of cells could not be obtained. Turpentine injected into the subcutaneous tissue caused necrosis and accumulation of a considerable number of leucocytes, but suppuration with softening of the tissue did not occur. A powder prepared by the method previously mentioned from the white fibrinous exudate at the seat of inoculation caused very weak proteolysis, when allowed to act at body temperature upon coagulated proteid in the presence of acid, but failed to digest in the presence of an alkaline reaction. Since previous experiments<sup>23</sup> have shown that the bone marrow of the dog contains that enzyme, namely, leucoprotease, which is characteristic of the polynuclear leucocytes, the proteolytic action of a suspension of cells of the bone marrow from the hen was tested in the presence both of acid and of alkali. The bone marrow removed from the bones was shaken violently in salt solution and forced through a fine sieve; the cells thus obtained were washed several times by centrifugalization and suspended in nine times their volume of salt solution. Five cubic centimeters of this suspension were allowed to act on coagulated proteid at body temperature in

<sup>23</sup> *Loc. cit.*

the presence of 0.2 per cent. acetic acid and of 0.2 per cent. sodium carbonate, and with the reaction of the medium unchanged. The experiments of Hedin and of one of us have shown that while the spleen of various mammals undergoes more active autolysis and digests foreign proteid more energetically in an acid medium, it contains at the same time no inconsiderable quantity of an enzyme which digests in the presence of alkali. The proteolytic action of a suspension of splenic cells from the hen was compared with the similarly prepared suspension of bone marrow from the same bird; for further comparison, a suspension prepared from the liver was used. In the following table the digestion of coagulated proteid produced by these suspensions is represented by centimeters of 1/10 N. acid.

	Bone marrow.	Spleen.	Liver.
With reaction unchanged	3.0 c.c.	3.45 c.c.	2.55 c.c.
With 0.2 per cent. acetic acid	10.7 c.c.	12.15 c.c.	8.6 c.c.
With 0.2 per cent. sodium carbonate	2.85 c.c.	3.05 c.c.	2.35 c.c.
Control	2.15 c.c.		1.8 c.c.

These figures show that the bone marrow of the hen, unlike that of the dog, fails almost completely to cause digestion of proteid in the presence of an approximately neutral or alkaline reaction, but causes active proteolysis in an acid medium. An enzyme similar to leucoprotease of the dog, if present, occurs only in very small quantity. The spleen likewise causes very trivial digestion in an alkaline or neutral medium, but is active in an acid medium; the control for the spleen which is lacking doubtless closely approximates that of the bone marrow. The liver which in an acid medium causes less digestion than bone marrow or spleen almost completely fails to digest in a neutral or alkaline medium. An enzyme which digests in an alkaline medium, and occurs in abundance in the organs of mammals is according to the foregoing observations almost wholly wanting in the tissues, notably in the blood-forming organs of the bird.

The absence in the blood-forming organs of the hen of this enzyme is apparently due to its absence in the polynuclear leucocytes. By injecting turpentine into the peritoneal cavity of the hen, a sterile inflammation results: in two experiments at the end of three

and of four days the cavity was found to contain yellowish fluid, but the intestines are loosely matted together by a white or pale greenish, gelatinous material in which are numerous opaque yellow spots. This exudate consists of fibrin holding serum in its meshes; the characteristic polynuclear leucocytes of the bird are present in large number. Bits of this fibrinous exudate washed with salt solution as free as possible of serum were suspended in acid, neutral, and alkaline solutions of which the volume was brought to five cubic centimeters by addition of .85 per cent. salt solution. After digestion at 37° C. during seven days the result of the experiment was as follows:

	Condition of fibrin.
With 0.2 per cent. acetic acid.	Fine powdery sediment.
With 0.1 per cent. acetic acid.	Much eroded.
In normal salt solution.	Unchanged.
With 0.1 per cent. sodium carbonate.	Unchanged.
With 0.2 per cent. sodium carbonate.	Unchanged.

While the experiment does not demonstrate with certainty that solution of fibrin in the presence of acid is due to enzymotic action, the result agrees with that obtained with bone marrow and demonstrates that autolysis of fibrinous exudate containing polynuclear leucocytes fails to occur in a neutral or alkaline medium. The fibrinous exudate obtained several days after injecting turpentine into the pleural cavity of the dog when washed free from serum undergoes complete autolysis, if immersed in 0.2 per cent. sodium carbonate and kept at body temperature.

#### CONCLUSIONS.

The inhibiting action of the blood serum upon the enzyme of the polynuclear leucocytes, leucoprotease, is exerted by the albumin fraction of the serum. The albumin fraction contains no proteolytic enzymes.

The globulin fraction of the serum contains no anti-enzyme for leucoprotease; it contains, on the contrary, an enzyme which digests proteids in a neutral or alkaline medium. This enzyme resembles leucoprotease which is present in the polynuclear leucocytes of an inflammatory exudate and in the bone marrow from which these cells are derived, and is doubtless identical with the similar enzyme

occurring in smaller quantity in the spleen. This enzyme which is present in the blood serum is held in check by its anti-enzyme, but the latter is in such excess that the serum as a whole is capable of checking the action of leucoprotease when added in considerable quantity.

Leucoprotease of one mammalian species is inhibited by sera of other mammalian species, but the anti-enzymotic activity of various sera differs; the anti-enzyme of the rabbit's serum is stronger than that of dog's serum, when tested either with dog's or with rabbit's leucoprotease. The co-existence in the rabbit of leucoprotease with feeble strength and anti-body of great activity may explain the absence in these animals of typical suppuration with liquefaction of tissues.

The serum of birds which have been tested, namely, pigeon and hen, almost completely fails to inhibit mammalian leucoprotease (of dog). The polynuclear leucocytes, the bone marrow and the spleen of the hen do not contain an enzyme resembling leucoprotease of mammals. The absence of anti-enzyme in the serum is associated with absence of a corresponding enzyme in the leucocytes.





HETEROTRANSPLANTATION OF BLOOD VESSELS  
PRESERVED IN COLD STORAGE.

By ALEXIS CARREL.

## HETEROTRANSPLANTATION OF BLOOD VESSELS PRESERVED IN COLD STORAGE.

By ALEXIS CARREL.

(From the Rockefeller Institute for Medical Research, New York.)

For the purposes of the transplantation of blood vessels in man and animal, a method which will suffice to prevent for a time the disintegration which extirpated tissues quickly undergo, would be of great value. With the view of retarding the occurrence of these cadaveric changes, I kept in cold storage for several days blood vessels resected from dogs; and afterwards I transplanted these vessels to cats. It is well known that the tissues of an animal do not grow, or grow hardly at all in an animal of another species. Nevertheless, these heterotransplantations were attempted with the aim of ascertaining whether the vessels, in spite of the toxic action of the cat's blood on the dog's tissue, could adapt themselves to the new conditions of life, and take over the function of the vessels removed. Five experiments were performed.

*Experiment I. Female Cat.*—A segment of the abdominal aorta located between the mouths of the renal and ovarian arteries was resected. The circulation in the lower part of the aorta was re-established by interposing a segment of the external jugular vein between, and suturing it to the cut ends of the aorta. This segment of the jugular vein had been extirpated from a dog seven days before and kept in an isotonic solution of sodium chloride at or about the freezing point. But the temperature of the refrigerator was not constant, and sometimes varied from 32° F. to 40° F. After the operation was completed, the pulsations of the femoral arteries and of the arteries of the hind limbs were normal. On the second day, the hind limbs became hyperæsthetic. On the third day, paralysis of the hind feet appeared, and a few hours later paralysis of the hind limbs was noted. At the same time, the pulsations of the femoral arteries completely disappeared. On the following days, the paralysis diminished progressively. Twenty-two days after the operation, the animal could walk almost normally. But the pulsations of the femoral arteries did not reappear. By direct examination it was found that the transplanted venous segment was surrounded by a thick layer of vascular connective tissue and its lumen was completely obliterated.

*Experiment II. Female Cat.*—A segment of the abdominal aorta of a large cat was resected and replaced by a segment of carotid artery which had been extirpated from a young dog twenty days before, and kept in an isotonic sodium chloride solution at about 32° F. Sometimes the temperature of the refrigerator rose for a few hours to 40° F., or even 44° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal. Forty-eight days after the operation, the animal was etherized and its abdomen opened. The pulsations were found normal in the abdominal aorta, and in the segment of carotid. The location of the anastomosis was marked by a slight hardening of the arterial wall. There was little reaction of the connective tissue around the transplanted vessel. The wall of the carotid appeared to be less elastic than that of the aorta. No dilatation of the transplanted segment was observed. The abdomen was closed after the examination, and the animal kept alive. Now, seventy-seven days after the transplantation, the pulsations of the femoral arteries are normal and the animal is in excellent condition.

*Experiment III. Male Cat.*—A large cat was subjected to an operation similar to the last one. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog three days before and kept in dog's defibrinated blood at 32° to 34° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal for thirty-four days. On the thirty-ninth day after the operation it was found that the pulsations of the femoral arteries had disappeared and that both anastomoses were occluded by a small deposit of fibrin. In the lumen of the transplanted segment was a soft clot of recent formation. The thrombosis appears to be due merely to an inclusion and proliferation of the external sheath of the carotid in the line of suture.

*Experiment IV. Male Cat.*—A small piece of the abdominal aorta of a large cat was resected, and a short segment of carotid artery was interposed between the cut ends. The arterial segment had been extirpated from a dog seventeen days before and kept in defibrinated dog's blood at 32° to 34° F. Ten days after the operation, the pulsations of the femoral arteries became weaker and disappeared completely. By direct examination it was found that the upper anastomosis was obliterated by a small deposit of fibrin. The upper part of the transplanted segment was filled with a clot of recent formation. The lower anastomosis was excellent. The endothelium and the wall of the segment of the carotid were apparently normal.

*Experiment V. Male Cat.*—An operation similar to the last one was performed on a large cat. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog four days before and kept in Locke's solution at 32° to 34° F. After the operation, the animal remained in normal conditions. He was killed six days after the operation. The union of the transplanted segment to the aorta was apparently perfect. There was no deposit of fibrin at the lines of suture and the wall of the segment of carotid artery was apparently normal.

## CONCLUSIONS.

The experiments show merely that blood vessels transplanted from dog to cat can act as arteries for seventy-seven days at least; and that having spent several days in cold storage does not interfere with their ordinary functions. The animals operated upon must, however, be kept under observation for several months, or, indeed, for several years, before any conclusion can be drawn concerning the practicability of this method of preservation and heterotransplantation of blood vessels.

## NUCLEIN METABOLISM IN A DOG WITH ECK'S FISTULA.

By J. E. SWEET AND P. A. LEVENE.

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All nitrogenous substances completely metabolized in the organism of mammals are removed by the urine in form of urea. Every other nitrogenous constituent of the urine is either a substance which has for one reason or another escaped its final transformation, or has not been metabolized by the organism at all. Under metabolism here, of course, is understood a set of chemical reactions which result, first, in maintenance of the integrity of the body's tissue and, second, in supplying it with calories sufficient to perform all the functions of the organism.

The quantity of nitrogen metabolized by the organism for the purpose of energy production is regulated primarily by the intake of nitrogenous food-stuffs, and by the quantity of reserve protein deposited in the organism. The quantity of nitrogen metabolized as a result of tissue deterioration for the purpose of maintaining its integrity is determined by the mass of the cells in the organism, by the amount of work which the tissues are called upon to perform, and by the intensity of the effort employed by the organism in performing the work. Thus, work of short duration, but of such intensity as would occasion a state of fatigue, brings about greater tissue deterioration than prolonged work of lower intensity.

Under normal conditions there exists a definite proportion between the two phases of nitrogenous metabolism, namely, between metabolism of nitrogenous food-stuffs of reserve protein on the one hand, and metabolism of cellular protein on the other. This proportion changes with the change of the plane of nutrition of the organism and with change of the quantity of protein in the food. However, conditions are conceivable in which it is made possible that the organism may be supplied with a sufficient amount of

energy by the sacrifice of the tissue integrity, or conditions may exist in which the tissue integrity is maintained by an unusually high consumption of reserve protein. Thus it is important to be able to trace separately the two phases of nitrogenous metabolism.

Analysis of the intake of food stuffs, a record of heat production, estimation of carbon dioxide and of urea removed by the organism furnish the data required to follow energy production. But one is confronted with considerable difficulty when he attempts to obtain information regarding the condition of the cellular elements of the tissues. This is due to the fact that deteriorated tissue elements are utilized for the purpose of metabolism in the same manner as the protein of the food or as the reserve protein. Their nitrogen is removed in the form of urea. Some components of the cellular elements, however, have a chemical composition distinctly different from that of the reserve protein. These substances, previous to their transformation into urea, undergo a cycle of chemical reactions which is different from that of the reserve protein; the intermediate products of their metabolism differ from those of the latter, and it is very probable that the mechanisms regulating the transformation of the two groups of substances are independent of one another. Indeed, in the light of this assumption one can readily explain the fact that transformation of special components of tissue elements varies considerably in different species, although transformation of simple reserve-protein occurs in an identical manner, and results in the same production of energy.

Among such components of tissue elements special interest is attached to the nucleins, since they constitute the principal substance of the nuclei of all cells. The attempt has been repeatedly made to ascertain the degree of tissue deterioration by analysis of the nuclear derivatives in the urine, the substances of special interest being uric and phosphoric acids. It has been established in recent years that uric acid is formed in the organism through the oxidation of purin bases, which are the principal components of nucleic acids. But uric acid is metabolized further in the organism of mammals, that is, it is oxidized and is finally removed from the organism in the form of urea. Uric acid detected in the urine represents only a fraction of the acid formed in the organism, namely, that part

which has escaped further oxidation. Hence satisfactory information regarding cellular disintegration cannot be gained by the investigation of uric acid output under normal conditions; but the results of uric acid analysis can be made valuable for the solution of the problem, if conditions are found under which the further transformation of uric acid can be prevented.

Clinical observations, and the studies of Pawlow, Nencki, Hahn and Massen especially, have suggested the probability that the liver is the principal organ concerned in the oxidation of uric acid. On the other hand, recent work of Weiner, Burian, Jones and Schittenhelm have shown that many other animal tissues possess the same power. It must be borne in mind that the Russian investigators were not engaged in the study of purin metabolism especially, and their observations were made at a time when uric acid formation was not yet clearly understood. The facts make a revision of the older work on purin metabolism very desirable.

The present investigation was carried out on a dog in which an anastomosis between the portal vein and the inferior vena cava had been successfully performed by one of us (Sweet). The purposes of investigation were: (1) To ascertain whether or not the output of uric acid was above normal, if the dog was maintaining nitrogenous equilibrium, and was keeping its original weight. (2) To ascertain whether or not the output of uric acid was increased markedly after administration of nuclein or of its derivatives (nucleoproteid of the mammary gland of the cow, nucleic acid of fish sperm, adenin sulphate, and thymin were employed). (3) To ascertain whether the animal possessed the power to metabolize thymin. (4) To ascertain whether or not the output of uric acid was increased on a diet poor in protein material, although containing carbohydrates and fat in quantities sufficient to supply the organism with the required amount of calories. (5) To ascertain whether or not the output of uric acid was increased during starvation.

The present communication represents the results of experiments performed on one dog, and cannot, therefore, serve as a basis for broad generalization. The work is communicated in its present incomplete state, because accidental circumstances render its im-

mediate completion unpracticable; the results already obtained are believed to be of interest.

*Methods.*—The urine was collected in three-day periods, the dog being catheterized at the beginning and at the end of each period. The receptacle contained a quantity of 10 per cent. solution of sulphuric acid to make up with the urine a solution of about 2 per cent. of sulphuric acid. No other preservative was added.

The nitrogen estimations were made by the Kjeldahl-Gunning method. The phosphorus was estimated gravimetrically, the urine being evaporated and fused with a mixture of potassium carbonate and sodium nitrate. Uric acid was estimated by the Leube-Salkowski method, ammoniacal silver chloride being used instead of the nitrate.

*Elimination of Uric Acid.*—Information regarding uric acid elimination by dogs with Eck's fistula is limited to the observations of Pawlow, Nencki, Hahn and Massen. Their dogs were fed on a mixed diet, no special attention being paid to the quantity of purin bodies in the food-stuffs, and no special care being exercised in collecting the daily quantities of urine. Moreover, since the dogs used by the Russian investigators developed the typical nervous attacks described by Pawlow, without further evidence the objection might be urged that the high uric acid output was occasioned by deterioration of the nuclear elements of the liver, or was secondary to the convulsive attacks.

In the present work the experiment was not begun until the nutrition of the dog after operation was so much improved that it maintained nitrogenous equilibrium. Furthermore, the dog was fed on cracker dust, plasmon, lard, and salt, to eliminate all purin in the diet, and had been on this diet for several weeks prior to the beginning of our records. It is, therefore, obvious that all the uric acid found in the urine must have originated in disintegration of tissue. The output of uric acid in this experiment was considerably higher than that of a normal dog.

*Feeding of Nucleoprotein.*—On two successive days the dog received, instead of plasmon, twenty-five grams of nucleoprotein of the mammary gland of the cow. As seen from the table, a slight increase in the uric acid output occurred, but as the nucleoprotein



## FEMALE MONGREL SPANIEL—OPERATION, MARCH 5, 1906.

Date.	Dog Received.	Weight	Total N-Intake.	N-Output.	N in Urine.	N in Faeces.	Total Uric Acid.	Ingested P <sub>2</sub> O <sub>5</sub>	P <sub>2</sub> O <sub>5</sub> in Faeces.	P <sub>2</sub> O <sub>5</sub> in Urine.	Amount of Urine.	Weight of Dry Faeces.
Apr. 27-28-29.	{ 75.0 g. Plasmon, 300.0 g. Cracker dust, ca. 35.0 g. Lard.	9250	12.891	14.140 g.	12.909	1.231 g.	0.240 g.	3.9045 g.	—	2.826 g.	1500	38.0 g.
30-May 1-2.	{ 75.0 g. Plasmon, 300.0 g. Cracker dust, ca. 35.0 g. Lard.	9400	"	14.867	12.18	2.687	0.492	"	1.3278 g.	2.254	2020	88.0
3-4-5.	{ 25 g. Plasmon, 300 g. Cracker dust + 50 g. Nucleoprotein.	9250	"	14.226	12.78	1.446	0.663	"	—	2.157	1700	45.0
6-7-8.	{ Plasmon, Cracker dust, Lard.	9250	"	12.559	11.2	1.359	0.230	"	—	2.113	1000	43.0
*16-17-18.	{ " + 1 g. Adenin sulphate.	9250	"	12.118	10.60	2.028	0.620	"	—	2.317	2100	65.0
19-20-21.	{ Plasmon, Cracker dust, Lard.	9150	"	14.631	13.8	0.831	1.050	"	—	3.156	3000	43.0
22-23-24.	{ " + 10 g. Nucleic acid.	—	"	15.13	13.37	1.766	1.301	"	2.519	3.947	3420	68.0
25-26-27.	{ Plasmon, Cracker dust, Lard.	9200	"	13.75	12.53	1.22	0.681	"	—	2.632	2710	58.0
28-29-30.	{ " " " "	—	"	13.804	12.64	1.164	0.721	"	—	2.644	2560	38.0
31-June 1-2.	{ " + 3 g. Thymine.	9200	"	12.512	11.22	1.292	0.707	"	—	2.408	3005	45.0
3-4-5.	{ Plasmon, Cracker dust, Lard.	9300	"	14.106	11.986	2.12	0.644	"	—	2.596	3020	58.0
6-7-8.	{ " + 6 g. Thymine.	9350	"	12.874	11.708	1.166	0.755	"	—	2.940	4425	43.0
9-10-11.	{ Plasmon, Cracker dust, Lard.	9400	"	12.602	11.401	1.201	0.796	"	—	2.517	2800	38.0
12-13-14.	{ " " " "	9550	"	13.542	11.835	1.617	0.840	"	—	2.690	2720	58.0
15-16-17.	{ " " " "	9650	"	14.068	12.513	1.555	0.797	"	—	2.382	2800	60.0
18-19-20.	{ " " " "	9600	"	14.079	12.609	1.380	0.773	"	0.9322	2.467	2650	48.0
21-22-23.	{ 195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	9700	3.042	9.467	8.808	0.659	0.917	0.774	—	1.846	2800	28.0
24-25-26.	{ 195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	—	"	7.638	7.140	0.498	0.972	"	—	1.622	3260	23.0
27-28-29.	{ 195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	10350	"	12.428	8.708	3.720	1.025	"	—	1.902	3590	285.0 †
30-July 1-2.	{ 195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	11200	"	10.824	7.382	3.442	0.857	"	—	1.294	2700	422.0 †
3-4-5.	{ Starvation.	8700	0.0	7.344	7.344	No faeces.	0.906	None.	0.0	1.518	3200	0.0
6-7-8.	{ " "	8350	0.0	6.65	6.65	No faeces.	0.746	None.	0.0	1.390	1450	0.0

75.0 g. Plasmon (3 days) = 8.835 g. N.; = 2.8725 g. P<sub>2</sub>O<sub>5</sub>. \* May 9-15 not worked out, owing to accidental loss of urine.  
 300.0 g. Cracker dust (3 days) = 4.056 g. N.; = 1.032 g. P<sub>2</sub>O<sub>5</sub>. † Faeces does not dry thoroughly, owing to large amount of sugar contained.

employed in the experiment had been extracted with alcohol and dried, its absorption and assimilation were perhaps somewhat unsatisfactory. Few experiments in which dogs have been fed on pure nucleoproteins are recorded, but the feeding of organs rich in nucleoproteins and purins in the experiments which have been reported have failed to cause any marked increase in the elimination of uric acid.

*Feeding of Adenin.*—One gram of adenin sulphate was administered the first day of the experiment. All earlier experiments in which dogs were fed on purin bases failed to show any increase in uric acid output. Only Minkowski<sup>1</sup> records an increased elimination following xanthin or hypoxanthin feeding, but he failed to obtain an increase with adenin. In the present experiment a slight increase in the uric acid output was noticed during the period of feeding; it was more marked in the following period. It is difficult to determine from this one experiment whether the rise was actually occasioned by the administration of adenin or occurred accidentally. Observations are recorded in which increase of uric acid appeared a few days after the administration of nuclear material (Camerer<sup>2</sup>). The increase in uric acid elimination during the period of adenin feeding, and during the following period was 0.3668 gram, an amount equal to 44.27 per cent. of the administered adenin. There was also noted an apparent retention of nitrogen during the period of the experiment, and an increase in the period next following, while during the latter period an increase in the uric acid elimination and diuresis were noted. All these findings may be explained by the action of adenin on the kidneys.

*Feeding with Nucleic Acid.*—The dog received in addition to its usual food ten grams of the sodium salt of nucleic acid of fish sperm. There was a marked increase of uric acid, an increase of phosphoric acid elimination and increased diuresis. The increase of nitrogen elimination did not exceed 0.8 gram. The nitrogen of this increase apparently had its origin in the absorbed nucleic acid, and corresponded to about 4.8 grams of nucleic acid. In reality, however, part of the increase in nitrogen elimination was occasioned by the

<sup>1</sup> *Arch. f. exper. Path. u. Pharm.*, 1898, xli, 375.

<sup>2</sup> *Zeitsch. f. Biol.*, 1896, xxxiii, 139; 1897, xxxviii, 206.

higher diuresis. According to the analysis of Levene and Mandel,<sup>3</sup> the nucleic acid employed in this experiment contained not more than 0.4 gram of purin bodies. The increase of uric acid elimination was about 0.2000 gram, which represents about 40 per cent. of the purin ingested with the nucleic acid.

In normal dogs an increase in the output of uric acid after nucleic acid feeding is recorded by Minkowski.

*Feeding of Thymin.*—Feeding experiments with pyrimidin bases have been performed by Stendel<sup>4</sup> with the purpose of studying the synthetic formation of uric acid in the living organism. Administration of thymin remained without influence on the output of uric acid. Stendel's efforts to recover the substance in the urine were unsuccessful.

In the present experiment, the dog received six grams of thymin, which was added to his food. No increase in the total output of nitrogen was noted; there was rather a slight nitrogen retention. On the other hand, a marked diuresis occurred, and the urine contained about 13.5 grams of the ingested thymin.

In order to obtain thymin, one liter of the urine was rendered acid by means of nitric acid and treated for pyrimidin bases by the Kossel-Jones method. The substance obtained in this manner was recrystallized out of very dilute sulphuric acid. A free crystalline base showing all the properties and the appearance of thymin was obtained. The substance had the following composition:

0.1372 grams gave 27.4 c.c. of nitrogen (over 50 per cent. potassium hydroxide) at  $t^{\circ} = 32.0^{\circ}$  C. and  $p = 756$  mm.

For  $C_4H_6N_2O_2$ :

Calculated  $N = 22.22$  per cent.

Found  $N = 22.96$  per cent.

Thus the greater part of the ingested thymin is eliminated by the kidneys; but it is difficult to form an opinion as to the extent of the decomposition in the organism, since it is possible that only part of the thymin had been absorbed, while another part may have suffered decomposition in the intestinal tract, through the action of bacteria.

<sup>3</sup> *Zeitsch. f. physiol. Chem.*, 1906, 1, 1.

<sup>4</sup> *Ibid.*, 1901, xxxii, 285.

Since it had been observed that the oxidation of thymine in the organism of the dog employed in this experiment was greatly impaired, an attempt was made to discover thymine in the urine obtained during the purine free periods, and also during the periods following the nucleic acid feeding. This was done with a view to establish the extent of nucleic acid decomposition in the organism. It was expected that thymine would be found in the urine, if considerable quantities of it were formed in the organism. From the experience of one of us (Levene<sup>5</sup>) with autolysis of animal organs, and also from that of Jones<sup>6</sup> and of Reh,<sup>7</sup> it is known that on disintegration of tissues, pyrimidine bases are formed. Nevertheless, neither during the period of nuclein free diet nor during that of the nucleic acid feeding, did the urine show any traces of thymine. One is, therefore, led to the conclusion that in the living organism nucleic acid either does not suffer complete disintegration, or disintegrates slowly, the small quantities of thymine thus formed being further decomposed.

*Diet with a Low Protein Content.*—The object of these experiments was to obtain further information regarding the factors which regulate the output of endogenous uric acid. Our present knowledge of endogenous uric acid is based on a very limited number of observations made on men. On the normal dog the study of nuclein metabolism has not been possible, since the intermediate products of decomposition of these substances are very readily transformed by the animal into urea. However, a dog with Eck's fistula possesses a lower degree of oxidizing power for purine bases than even man. Thus, it is seen, after feeding adenine that nearly 45 per cent. of the substance was eliminated with the urine in form of uric acid. Burian and Schur<sup>8</sup> found in men, after administration of hypoxanthine, a figure approaching this, and one less than half so high after feeding other purine bodies. It is believed by most observers that the output of endogenous uric acid remains practically constant and is totally independent of the quantity and of the quality of the food taken.

<sup>5</sup> *Zeitsch. f. physiol. Chem.*, 1901, xxxii, 546; 1903, xxxvii, 521; 1904, xli, 402.

<sup>6</sup> *Ibid.*, 1904, xlii, 35.

<sup>7</sup> *Hofmeister's Beiträge*, 1903, iii, 569-573.

<sup>8</sup> *Pflüger's Arch.*, 1900, lxxx, 342.

True, Burian and Schur,<sup>9</sup> the principal advocates of this view, considered it necessary to point out that extreme changes in the quantity or in the character of the food may alter the output of endogenous uric acid. Excessive intake of food, which taxes the gastrointestinal glands beyond normal, may result in an increased uric acid elimination, while on the other hand, starvation results in a decreased uric acid output, since it lowers the intensity of general metabolism. Folin<sup>10</sup> much more emphatically expresses the view that the output of uric acid is not invariably the same for an individual, as Burian and Schur<sup>11</sup> claim.

Folin arrives at this conclusion: "When the total amount of protein metabolism is greatly reduced, the absolute quantity of uric acid is diminished, but not nearly in proportion to the diminution in the total nitrogen, and the percentage of uric acid nitrogen in terms of the total nitrogen is, therefore, much increased." L. B. Mendel<sup>12</sup> states, "When the total amount of protein metabolism is greatly reduced, the endogenous output of uric acid is diminished, though this is not the case within ordinary ranges of diet."

A reduction of the intake of albuminous food-stuff should occasion for a time an increased disintegration of the body proteins, and with it a greater destruction of the cellular elements of the tissues. This, in its turn, should result in a greater formation of uric acid. Therefore, it seemed to us probable that the diminished uric acid output, under the conditions indicated by Folin, Mendel, and others, was caused, not by diminished formation, but by more complete combustion of the purin bodies. *A priori*, it might be expected that the organism utilized more completely nitrogenous substances of non-protein nature, when there was a lack of protein in the food. If this assumption were correct, one should find under these conditions an increased elimination of uric acid when the power of the organism to consume purin bodies is diminished. In our experiments, indeed, the diminution of the protein intake and its substitution by fat and carbohydrate (so that the intake of

<sup>9</sup> *Loc. cit.*

<sup>10</sup> *Amer. Jour. of Physiol.*, 1905, xiii, 87.

<sup>11</sup> *Loc. cit.*

<sup>12</sup> Harvey Lecture, *Journal of the American Med. Ass.*, 1906, XLVI, 843, 944.

calories was not altered) was followed by an increase in the uric acid elimination. It was expected that the organism would, on a continued low protein diet, finally adjust itself to the condition and preserve the integrity of the remaining cellular elements. In our dog, the uric acid elimination suffered a marked fall during the fourth low protein period.

*Starvation.*—In order to understand fully the influence of diet on the output of endogenous uric acid it was considered necessary to ascertain the influence of starvation on uric acid elimination. It was originally planned to have the starvation period precede the period of low protein diet. Since fasting might prove fatal to the dog, it was thought safer to change the order of the experiments. During the first starvation period, the uric acid elimination showed a rise, as compared with the preceding period. The output, however, was not as high as during the early period of low protein diet. Comparing the starvation periods with the periods of diminished protein diet, one gains the impression that the uric acid output was higher during the latter experiment. One must, however, bear in mind that this experiment preceded starvation and the continued low protein diet caused a considerable diminution in the mass of the cellular elements of the body. On the other hand, it is also possible that the absence of activity of the digestive glands is the cause of the lower output of uric acid during starvation.

*The Period Following Starvation.*—After the second starvation period the animal received the usual diet, consisting of cracker dust, plasmon, and lard. It ate all the food given, but on the next day refused to eat, and later developed the typical symptoms described by Pawlow. Once, typical epileptic convulsions were observed by the writers. The animal remained for forty-eight hours without food, and showed a tendency to recover. It refused the usual food—plasmon, etc.—and also milk, and was then given boiled meat which it ate ravenously, apparently improving. However, after eighteen hours, it again relapsed into the nervous condition described, and was found dead on the morning of the fifth day after the end of the starvation period.

The autopsy showed a condition of extreme emaciation. Aside from cachexia, the organs of the body presented a normal appear-

ance. The liver was possibly of smaller size than usual, but showed no cirrhotic or other lesions. The veins of the abdominal viscera were filled with an injection mass, the vena cava having been tied above the diaphragm, and the site of the anastomosis was minutely dissected; no collateral branches, which sometimes carry portal blood around the ligature about the portal vein, were found. The opening of the fistula was large and perfectly formed.

*On the Diuretic Action of Nuclein Derivatives.*—A review of the tables clearly shows that a more or less pronounced diuresis follows the administration of nuclear material. The tables of Burian and Schur,<sup>13</sup> and those of Minkowski indicate the occurrence of this phenomenon. In the experiments of these investigators, increased diuresis followed only the administration of nucleins, or nuclein containing tissues, but did not follow the administration of the free purin bases. In our experiments diuresis was less pronounced after the administration of adenin. It is worthy of note, however, that the administration of thymin caused the most pronounced diuresis. As is well known, the methylated dioxypurins possess a much higher diuretic action than other purin derivatives, and it seems probable that the methylated dioxypyrimidin also possesses a high diuretic action. This question, however, can be answered only after further study.

<sup>13</sup> *Loc. cit.*





## ON THE ELECTRICAL CHARGE OF THE NATIVE PROTEINS AND THE AGGLUTININS.

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In a previous paper<sup>2</sup> it was shown that the particles of both toxin and antitoxin wandered under the influence of an electric current toward the cathode and that the reaction (acidity or alkalinity) of the solvent did not influence the direction of migration. Since Hardy<sup>3</sup> and Pauli<sup>4</sup> demonstrated that the proteins which they used were amphoteric, *i. e.*, that they pass toward the anode in an alkaline medium and to the cathode in an acid one, there has been a tendency to generalize by assuming that all proteins behave in this manner. If such were the case, we pointed out, it would follow from our experiments that toxin and antitoxin are not true proteins. At the same time, however, we mentioned that from the few experiments in which this question had been considered, the protein matter of the broth or serum seemed in every instance to travel with the toxin or antitoxin toward the cathode. Further experiments have confirmed this result. It was also shown that the protein of normal horse serum and of non-toxic broth travels toward the cathode. Hence our work offers as yet no evidence either for or against the view that toxin and antitoxin are non-protein in nature.

We maintain that the results which Hardy and Pauli obtained, working with denaturalized proteins, are in no wise applicable to the native proteins, but that these carry a distinct electrical charge and are not amphoteric. We are here in accord with Iscovesco<sup>5</sup> and his co-workers, who investigated the charge of colloids con-

<sup>1</sup> Assisted by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> Field and Teague, *Journal of Exper. Med.*, 1907, viii, 86.

<sup>3</sup> *Jour. of Physiol.*, 1899, xxiv, 288.

<sup>4</sup> *Hofmeister's Beit.*, 1906, vii, 531.

<sup>5</sup> *Compt. rend. Soc. biol.*, 1906, lxi, 195, 355, 378, 470, 568.

tained in various body fluids. Their method consisted in treating the fluid with electro-negative (arsenic sulphide) and electro-positive (ferric hydrate) inorganic colloids and their conclusions were based upon the fact that colloids of opposite sign when brought together form precipitates. Thus they found that the peritoneal fluid of the horse contains only electro-positive colloids, while the pericardial fluid contains those of both signs; that blood plasma contains both positive and negative albumins with positive and negative globulins, whereas the serum contains only the positive globulin along with albumins of both signs; that the fluid of a tubercular abscess deprived of its leucocytes contains only electro-negative colloids; that the amniotic fluid contains both positive and negative albumins, but only negative globulins. From these experiments Iscovesco concludes that there are no colloids which do not bear a distinct electrostatic charge.

Since our method gave no indication of the presence of an electro-negative albumin in normal serum, we are inclined to believe that Iscovesco by his manipulations produced a change in sign of the charge carried by certain proteins and that all of his findings are therefore not applicable to the proteins originally present in the fluids he investigated.

In our previous work with tetanus toxin we investigated only the tetanospasmin and its antibody; we have since shown by testing the agar extracts for their lytic or antilytic action on horse cells that both tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current. Having determined the electrical charge of toxin and antitoxin, we next applied the same method to an investigation of the agglutinins.

The agar was divided into one centimeter lengths; the agglutinin was found to have traveled seven centimeters into the cathode agar, the anode agar remaining free of agglutinin. The first centimeter length was extracted with five cubic centimeters of water and this extract would still agglutinate at a dilution of 1:100.

The specific agglutinins investigated travel toward the cathode. These results are diametrically opposed to those of Biltz, Much and Siebert,<sup>6</sup> who are the only workers, so far as we know, who

<sup>6</sup> *Zeit. für diätet. und physikal. Ther.*, 1905, viii, 19.

have investigated this subject. They passed a current through lacto-serum contained in a U-shaped tube for from one half to one hour, and found that the fluid around the anode agglutinated at 1:20, that around the cathode not at all, and that from the middle of the U-shaped tube at 1:8. Normally the serum agglutinated at 1:4. They state that after the passage of the current the fluid from around the anode was 1/10 normal acid. We would expect this amount of acid to agglutinate at approximately 1:20, since 1:200 represents about the flocking limit of hydrochloric acid for bacteria.

As stated in a previous article, we took special precautions to eliminate the products of electrolysis. However, to show conclusively that it was the specific agglutinin, and that alone, which was responsible for the agglutination in our experiments, the extracts were also tested against other bacilli than those which were agglutinated by the serum under investigation.

TABLE I.

STRENGTH OF ELECTRIC CURRENT 110 VOLTS;  $\frac{1}{2}$  TO 1 MILLI-AMPERE.

Serum agglutinating the typhoid bacillus at 1:2000. Current passed for six hours.

Organism.	Cathode Agar cm. Lengths.											Anode Agar.	
	1	2	3	4	5	6	7	8	9	10	11-20	1-10	11-20
B. typhosus.	+++	++	+++	+++	+++	++	+	0	0	0	0	0	0
B. coli.	0	0	0	0	0	0	0	0	0	0	0	0	0
Shiga's bacillus.	0	0	0	0	0	0	0	0	0	0	0	0	0
Para typhoid b.	0	0	0	0	0	0	0	0	0	0	0	0	0
Biuret react.	+	+	+	+	+	+	trace						

NOTE.—As one centimeter lengths of the anode agar showed no agglutinin in repeated experiments we have here tested extracts from ten centimeter lengths.

If the agglutination were due to the presence of products of electrolysis we would expect the other bacilli to be agglutinated as well as typhoid. Such, however, was not the case. Hence, we believe that we have shown conclusively that the agglutinins travel toward the cathode.

It has been shown by Bechhold<sup>7</sup> and Buxton, Schaeffer, and Teague<sup>8</sup> and others that bacteria move toward the anode under

<sup>7</sup> *Zeit. physik. Chem.*, 1904, xlviii, 385.

<sup>8</sup> *Ibid.*, 1906, lvii, 47.

the influence of an electric current, that is, they carry a negative charge.<sup>9</sup> Our findings with regard to the agglutinins is therefore especially interesting, since it shows that in the phenomenon of agglutination we have the combination of an electro-negative suspension with an electro-positive colloidal solution. Since ions of opposite sign are essential for a chemical reaction and colloids of opposite sign when brought together form precipitates, our results harmonize with both the chemical and the colloidal view of the phenomenon.

Bacteria which have been saturated with agglutinin and then washed in a number of changes of water until the wash water contains no more agglutinin were placed in the cell and after eight hours the agar was tested for agglutinin. A small amount was found in the cathode agar showing that under the influence of the electric current the agglutinin bacteria combination was disassociated and that the agglutinins passed to the cathode. Bacteria have been disassociated from agglutinins by other means,<sup>10</sup> but so far as we are aware, this is the first time that disassociation has been affected by means of the electric current.

#### CONCLUSIONS.

1. Tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current.
2. The specific agglutinins are electro-positive.
3. The proteid matter of serum is not amphoteric but travels toward the cathode whether its reaction be acid, neutral, or alkaline.
4. The bacteria-agglutinin combination may be disassociated by means of the electric current.

<sup>9</sup> Cernovodeanu and Henri (*Compt. rend. Soc. de Biol.*, 1906, lxi, 200) claim that dysentery bacilli travel toward the cathode but we have not found this to be the case.

<sup>10</sup> Quoted by Eisenberg in *Cent. f. Bakt.*, 1906, xxxi, 540 are the following: Joos (if fresh bacilli are added to agglutinated bacilli, which have been previously washed free from serum, the former are agglutinated), Landsteiner and Jagio and Landsteiner and Reich (dissociation at high temperatures).

## THE TRYPANOSOMES OF MOSQUITOES AND OTHER INSECTS.\* †

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## INTRODUCTION.

IN the paper on "Bird Trypanosomes"<sup>1</sup> we expressed the opinion, in view of the ease with which trypanosomes can be cultivated in the test-tube, that it is reasonable to suppose the same result may take place in the mosquito. That is to say, the few trypanosomes which chance to be present in the blood sucked up by the mosquito may rapidly multiply in the stomach of the insect and give rise to rich cultures similar in many respects to those met with in the test-tube. This view, based entirely upon the cultural characteristics of the bird trypanosomes, has an immediate bearing, not only upon the question of the transmission of trypanosomes, but also upon that of the relation of flagellates to certain intracellular parasites, as claimed by the late Dr. Schaudinn, and for that reason it was desirable to obtain, if possible, an experimental confirmation.

The obvious procedure would be to allow mosquitoes which had been raised from eggs in the laboratory, to feed on birds having a pure trypanosome infection. On subsequent examination such mosquitoes should show cultural forms in the gut, if the above view was correct. Unfortunately, on account of the difficulty of procuring and keeping alive wild birds the choice of birds for these experiments was limited to the sparrow and canary. In our previous work we had found trypanosomes in sparrows, but only in a very small percentage of the birds examined. We hoped, however, by means of cultures of the bird trypanosome to secure pure infections of sparrows and canaries, and with this object in view, a large number of sparrows were examined during the summer of 1905, microscopically and culturally, but with negative results. The failure to obtain infected sparrows and the inability to work with *Tr. paddae* which, as shown by Thiroux, is admirably adapted for laboratory infection, compelled us to abandon this plan and resort to a different procedure.

The rather common occurrence in birds of trypanosomes, and of cytozoa, suggested a study of the flagellates in "wild" mosquitoes. If our view was correct, then it might be possible to find in the gut of such mosquitoes cultural forms of bird trypanosomes. As will be shown, flagellate infection of mosquitoes is quite common, but the forms met with do not correspond to any one of the numerous strains

<sup>1</sup> *Jour. Infect. Dis.*, 1905, 2, p. 303.

which we cultivated from birds. Previous observers had already described such organisms in the gut of insects, but their source or nature was far from being understood. They might be derived (1) from the trypanosomes of the blood of birds or other animals; or (2) they might be stages in the life-cycle of cytozoa, as held by Schaudinn; or (3) lastly they might be harmless, non-pathogenic parasites peculiar to the insect and in nowise related to either of the two types mentioned above.

It is plain that each of these three possibilities must be considered in order to arrive at a definite conclusion regarding the flagellates found in the gut of sanguivora, such as mosquitoes, flies, fleas, lice, leeches, etc. A mere morphological study is incapable of deciding for certainty between the three conditions. Even when supplemented by animal inoculations, effected either by the bite of the mosquito, etc., or by injection of suspensions of the insects, the result is still open to question. For it is conceivable that the mosquito or other host may have a double or even triple infection, and hence the result obtained (i. e., appearance of cytozoa) may be due to an unrecognized stage and not to the flagellates actually seen. The recent work of Edm. and Et. Sergent on the transmission of the halteridian infection of pigeons affords a demonstration of the existence of such a stage. It is true that in proteosoma infection, and in human malaria this method of investigation has given most satisfactory results, but when applied to flagellate studies it may prove, and undoubtedly has proven, misleading. The relation of cause and effect in such problems can be brought out with the least uncertainty by the aid of pure cultures of the trypanosomes in question, and this method of study, so well established in bacteriology, should be applied as far as possible to the solution of questions in protozoology. By this means we have already shown, contrary to Schaudinn, that the trypanosomes of birds are in nowise related to the hemocytozoa. The applicability of this method of research to insect trypanosomes will be seen from the results of this study.

Our investigations upon the mosquito trypanosomes were made during the summers of 1905 and 1906. They show that the flagellate infection of mosquitoes is of common occurrence, and that several distinct species are to be found. It has been possible to grow these

organisms in mixed and even in pure culture, and to show that they are closely related to, and indeed actually are, true trypanosomes. The forms which have been obtained in the culture-tube are similar to, and even identical with, those found in the digestive tube, thus showing that the forms met with *in vivo* are in reality cultural forms, as we have contended. This fact goes to show the absurdity of the contention that cultural forms represent degeneration or involution types. The cultivation method has enabled the differentiation into two species of forms heretofore regarded as belonging to one organism. Furthermore, the inoculation of various birds and mammals with such cultures was not followed by the development of intracellular parasites, or by the appearance of trypanosomes in the blood, which would seem to indicate that the mosquito flagellates are parasites peculiar to this insect.

#### PREVIOUS OBSERVATIONS.

Before proceeding to a detailed account of our work, it is desirable to present, as briefly as possible, a résumé of the observations which have been made up to the present time regarding the presence of flagellates in the guts of various insects and also of leeches. It will be seen that flagellate infection of the digestive tube is not limited to the sanguivora, but that it may occur in insects that do not feed or have not fed on animals.

The earliest observations upon the presence of flagellates in mosquitoes were made by Ross, in 1898, in India. Recently (1906) he has called attention to these early studies, and there can be no doubt but that the forms seen by him were the same as those which Léger subsequently described as *Crithidia fasciculata*. In various species of *Culex*, principally *C. fatigans*, he found "sporangia" or radiating clusters (rosettes) of small active bodies, which under water separated into thousands of flagellulae. These were about  $8\mu$  by  $2\mu$  in size, with numerous minute black points in their substance, and had a single long flagellum. Another form, which he designated as amoebulae, he now considers as transitional, dividing forms corresponding to the truncated *Crithidia*. Apparently, the long *Herpetomonas* which will be described later was not encountered. The forms mentioned were also observed by him in *Anopheles*, but not in *Stegomyia*. They occurred in the larva, pupa, and imago; at times, in a considerable proportion of the insects examined. It is interesting to note that Ross during his work on *Proteosoma* found the flagellates but once in mosquitoes, although these were fed on birds many of which contained *Halteridium* as well as *Proteosoma*. This fact, as well as their presence in all three stages of the insect, goes to show, as Ross points out, that they had been already present in the insects before these were fed on the blood. He very correctly concludes that there is little reason



for supposing that the *Crithidia* are developed from cytozoa, and he expresses the belief that they are derived from organisms contained in the intestines of the larva and probably swallowed by it. He further points out the probability that Schaudinn's mosquito flagellates were merely *Crithidia* which were already present in the insects with which he worked.

The next observation is that of Durham, who, in 1900, examined a specimen of *Stegomyia fasciata* which had fed the night before on a small bat. The blood in the mosquito contained abundant trypanosomes whose shape was quite different from the usual ones in rats, nagana, etc. The presumption was that these trypanosomes came from the bat, but this, owing to its death and rapid decomposition, could not be verified with certainty. Although flagellates, coccidia-like bodies, etc., were found from time to time in the eighty mosquitoes which were dissected, this was the only instance in which trypanosomes were found. Nothing further is stated regarding the flagellates mentioned.

Chatterjee, in 1901, while dissecting different *Anopheles*, found in the abdominal cavity of one of them an organism which appeared to him to be closely related to the trypanosome of surra. It was actively motile and possessed a fine flagellum, longer than the body, and an oval nucleus. Not having access to the original paper, it is impossible to say whether this form belonged to *Crithidia* or *Herpetomonas*.

In the same year Christophers noted the presence of swarms of a flagellate organism in the rectum and throughout the hind- and mid-gut, in a large proportion of *Anopheles* and *Culex*. The illustration given by him (Ref. 13, Fig. 3, Plate V) is suggestive of *Crithidia*. The presence of these parasites is also mentioned by Stephens and Christophers.<sup>62</sup> In this connection it may be stated that from recent private communications of Captain Patton and also of Captain Christophers we learn that a large percentage of mosquitoes at Madras have *Herpetomonas* and *Crithidia*. Christophers writes that he has found developmental or immature forms of *Herpetomonas*, clustered about the pylorus, in the larvae of *C. fatigans*.

An important study on the flagellates of mosquitoes was published by Léger in 1902. In the gut of *Anopheles maculipennis* he found a peculiar flagellate, presumably the same as that observed previously by Ross and Christophers, which on account of certain characteristics he placed in a new genus, to which he gave the name *Crithidia*. He classed it among the Cercomonadina, intermediate between *Herpetomonas* and *Trypanosoma*. The distinction between the two genera, *Herpetomonas* and *Crithidia*, was based largely upon the difference in size of the adult, free, or monadian forms. It will be shown presently that Léger failed to recognize the possibility of double infection in insects, and that he has at times included in one description two distinct species.

In his critical study of the blood flagellates, Lühe points out that the difference in size of the adult, free, or monadian forms is hardly sufficient to justify the creation of a new genus. Lühe, however, would characterize the genus *Crithidia* as possessing a single flagellum with the blepharoplast situated near the nucleus, whereas the true *Herpetomonas* has a double flagellum (as described by Prowazek for *H. muscae-domesticae* and *H. sarcophagae*) and a terminal blepharoplast. This characterization of the two genera is open to question, as will be shown later on. It should be borne in mind, however, that the distinctions between *Trypanosoma*, *Herpetomonas*, and *Crithidia* are at the best highly fragile.

It is interesting to note that Léger raised the question, in view of the way in which *Anopheles* fed, and the analogy of the elongated forms to trypanosomes, whether the

*Crithidia* did not represent a developing stage of some flagellate hematozoon, more especially the trypanosomata of vertebrates. This question is as yet unanswered, although it is probable that the *Crithidia* are parasites peculiar to the mosquito and are not immediately derived from the trypanosomes in the blood of other animals. As will be seen, Schaudinn has endeavored to show that these, or very similar forms, represented stages in the development of the *Halteridium*.

Léger designated the flagellate found by him in *Anopheles* as *Crithidia jasciculata*. He described two types with transitional forms one to the other. The first, or gregarine-like, or resting form, is more frequent. It appears as a short, thick oval, resembling a grain of barley. It is slightly flattened, and the anterior end, which carries a whip as long as the body, is truncated. These smaller forms measure 3-4  $\mu$  in length, but may attain 6-8  $\mu$ . The anterior end is slightly curved or may be indented. The body is hyaline or faintly granular, and at times shows one or more clear spots resembling vacuoles. The whip starts at the centrosome or blepharoplast which lies in front or to the side of the nucleus. The division is longitudinal and corresponds to that of trypanosomes. Rosette formation is common, and the small cells may be united in masses or in radiating bundles which are attached to the intestinal wall.

The second, termed the monadian or free form, is an elongated cell, 8-14  $\mu$  in length, and resembles exactly small trypanosomes. The anterior pointed end is drawn out along the whip, and the cell shows a rudimentary undulating membrane. As mentioned above, Léger held that the length of the free form afforded the chief distinction between this genus and that of *Herpetomonas*.

Léger and Duboscq in the same year found this organism, either as the free cells or in massive rosettes, in the intestines of *Anopheles* larvae, as well as in hibernating adults.

The work of Schaudinn, in 1904, is of especial importance, for, no matter what may be the final interpretation of his observations, it has served to call attention to the flagellates present in the digestive tract of insects and other sanguivora, and in so doing has stimulated inquiry, not only as to the relation of these forms to the blood protozoa, but also as to the mode of transmission of the pathogenic protozoa in general. As is well-known, he described the formation of trypanosomes and so-called spirochetes in the common mosquito, *Culex pipiens*, which had previously fed on owls infected with intracellular organisms (*Halteridium* and *Leucocytozoon*) and trypanosomes. In view of the fact that a summary of this extensive paper was given in our paper on "Bird Trypanosomes," it is hardly necessary to repeat it here. It was pointed out in that paper that the mosquito flagellates of Schaudinn were probably derived from the bird trypanosomes which had multiplied in the digestive tube of the insect. As will be seen, the present study offers another and more plausible explanation of the origin of Schaudinn's flagellates.

It is important to note, as Schaudinn himself has pointed out, that the forms of *Trypanosoma noctuae*, as found and described in the mosquito, show the greatest resemblance to the *Crithidia* of Léger. Indeed, because of the gregarine-like, resting condition and the mode of multiplication, he inclined to the belief that the *Crithidia* represented a developmental stage of a closely related parasite. Notwithstanding the fact that Schaudinn recognized this similarity, there is no evidence, as Ross rightly asserts, that he excluded the presence of *Crithidia* in the mosquitoes used in his experiments. It is a striking fact that, while he refers to Léger's observation on *Crithidia*, he makes no mention of the presence of the same or similar forms in the *Culex pipiens* with which

he experimented, and which were presumably captured at large. From what is now known regarding the distribution of *Crithidia* and *Herpetomonas* in mosquitoes, we must recognize a grave source of error which Schaudinn apparently failed to take into consideration. It will be remembered that he was able to find trypanosomes in only about 10 per cent of the mosquitoes used in his experiments. As will be seen, we have found flagellates in as many as 15 per cent of the mosquitoes captured during one season. This fact itself is very suggestive of the nature of the organisms observed by him. It is our belief that Schaudinn's *Tr. noctuae* is in nowise related to the owl halteridium, and that it either corresponds to *Crithidia fusciculata* or to a mixed *Crithidia* and *Herpetomonas*. Furthermore, the *Spirochaeta Ziemanni* which he later recognized as a trypanosome and not a true spirochete, would seem to correspond to a *Herpetomonas* like our *Tr. culicis* or to *Tr. avium* were it not for the recent observation of Töpfer regarding the presence of spirochetes in owls. This work, as will be shown later, throws new light upon the nature of *Sp. Ziemanni*.

In the same year (1904) Edm. and Et. Sergent, working in Algeria, repeated the work of Schaudinn and obtained essentially the same results.<sup>1</sup> That is to say, flagellates were found in 28 per cent (14 out of 50) of *Culex pipiens* which were examined 36-48 hours after having been fed on an owl (*Athene noctua*), infected with halteridia. This large percentage of infected mosquitoes, as compared with Schaudinn's 10 per cent, is of no special significance, since we have repeatedly met with small batches of "wild" mosquitoes in which one third or more showed flagellates. The Sergents state explicitly that they had never met with similar forms in the stomachs of mosquitoes which had not fed on infected blood.

This general statement, in view of the widespread natural infection of mosquitoes, is difficult to understand unless it be assumed that these control insects were examined directly—that is, without having been fed upon a clean animal. It is perfectly obvious that a rigid control test demands that the mosquitoes be fed upon the blood of a clean, or non-infected bird, or even mammal, in order to allow the flagellates, if any are present, to multiply. The fact that the mosquitoes used in these experiments were raised from larvae in the laboratory does not, as is now known, exclude a natural flagellate infection of the adult insect. This fact they themselves recognized in the following year, as will be shown presently.

With the mosquitoes which had fed upon the infected owls the Sergents were able to produce a halteridium infection in young owls which had been raised in the laboratory and were presumably free from parasites. Thus, an owl injected with the stomach contents (containing trypanosomes) of a mosquito that fed on an infected bird two days before, developed halteridia 11 days later. Similarly, a second owl which received the stomach contents of two mosquitoes showed halteridia three days

<sup>1</sup> The forms which the Sergents identified as "indifferent" trypanosomes measured  $25\ \mu$  in length by  $3\ \mu$  in width. The larger ones were  $36\ \mu$  long. This is given as the total length including the flagellum, the latter being as long as the body. The "male" trypanosomes were small, on an average  $14-15\ \mu$  in length, and this presumably includes the whip. The deeply staining, granular "female" forms were rare. They note a slight difference between their trypanosomes and those of Schaudinn in that their forms were "plus effilés, moins renflés" than those figured by the latter. This fact, taken into consideration with their description of the indifferent, male and female, forms, makes it probable that they were dealing with *Herpetomonas*, probably the same as our *Tr. culicis*. On the other hand, Schaudinn's figures (Figs. 1, 2, and 4) show a rounded posterior end, and in this respect resemble *Crithidia*; in other regards, like the presence of an undulating membrane and posterior bodies, they suggest a *Herpetomonas*. The *Herpetomonas* only rarely shows an enlarged, rounded posterior end, as will be shown later in connection with the involution forms of *Tr. culicis*.

later. On the other hand, a third owl which received an injection of the stomach contents of three mosquitoes failed to become infected (1). In another experiment six mosquitoes, fed a month before on an infected bird, were allowed to bite a young owl, which soon developed an infection. Two other owls bitten by these same mosquitoes or by others, failed to show halteridia.

The Sergeants looked upon their work as a crucial experiment supporting Schaudinn's view as to the relation of *Tr. noctuae* to the halteridium. It must be confessed, however, that these tests were by no means as rigid as they supposed; for, although they employed mosquitoes raised from the larval stage and made use of young birds taken from the nest, and found presumably to be free from infection, they clearly overlooked several possible sources of error. In the first place, the owls upon which the mosquitoes fed may have been infected with trypanosomes as well as halteridia. They themselves recognized the presence of a trypanosome in 1 out of the 3 or 4 infected owls with which they worked, and it is not unlikely that this flagellate was present in the others, though in very small numbers. It is therefore possible that the mosquitoes which fed upon these owls developed a double infection, trypanosomal and halteridian, the latter being readily transmitted by an unrecognized stage, whereas the former gave rise to a "culture" of the flagellates in the digestive tube. An error of this kind can be obviated by demonstrating that the birds are *culturally as well as microscopically* free from trypanosomes.

A second and perhaps even more important factor which must be taken into consideration is the fact of the not infrequent presence of trypanosomes in the larval and pupal stages, and hence in adult mosquitoes before these have fed upon the infected bird. It is evident that the use of mosquitoes which have been raised in the laboratory from the eggs does not insure the exclusion of flagellate infection of the intestinal tract of these insects.<sup>1</sup> This fallacy can be guarded against by means of a control experiment in which a large enough number of "raised" mosquitoes are fed upon non-infected animals. In this way it should be possible to show whether or not the percentage of flagellate infection is greater in the mosquitoes which fed upon infected birds, and whether such flagellates are identical with or different from those naturally present in the stomach.

A third and equally important objection is based upon the apparent absence of control inoculations with the stomach contents, free from trypanosomes, of mosquitoes fed on infected birds. In Schaudinn's work 90 per cent of the mosquitoes thus fed showed no flagellates, while with the Sergeants the percentage was not as high, only 72 per cent. What would be the result if the stomach contents of these mosquitoes was injected into clean owls? There is no record that such experiments were made; and yet the need of a control test of this kind is perfectly obvious; for, if the halteridian infection is due to something other than flagellates, it should be possible to secure positive results with such trypanosome-free mosquitoes.<sup>2</sup> On the other hand, the failure

<sup>1</sup> As will be seen from their work of the following year, reference to which is given below, they succeeded in finding a *Herpetomonas* in 11 per cent of mosquitoes raised from the larval stage in the laboratory.

<sup>2</sup> The possibility of the presence of an unrecognized stage of halteridium in the mosquitoes is seen from the recent work of the Sergeants on the transmission of this parasite to pigeons. After feeding some *Hippoboscides* (*Lynchia maura*) on infected pigeons in Algeria, the insects were taken to Paris and placed on clean pigeons. The latter became infected after a period of incubation of 34-38 days. Intravenous injections of suspensions of *Lynchia* caused infection after 28-29 days; and a Berkefeld filtrate infected after 36 days. This long period of incubation, when compared with that noted in owls after injection of mosquito trypanosomes, lends color to the belief that these birds had an unrecognized latent infection. Trypanosomes were apparently not present in the infected *Lynchia*.

to obtain infection in such an experiment would be an argument in favor of Schaudinn's views.

Another source of error which must be rigorously guarded against is the possibility of the existence of a latent halteridian infection in the owls seemingly free from parasites. The infection of owls and other birds very commonly takes place before they leave the nest. This fact was first pointed out by Danilewsky, and we have repeatedly made similar observations. Hence, nestlings cannot be said to be free from infection unless repeated examinations are made *extending over a period of several weeks* to allow for the maximum period of incubation. A very good illustration of latent infection is sometimes observed in sparrows which receive an injection of proteosoma. Although at first apparently free from parasites, they may develop halteridia in the course of a week, along with the proteosoma. The halteridia were not introduced by the injection, for it is not possible to infect birds in that way. The statement of Schaudinn that the halteridian infection of owls can be transmitted from owl to owl by injections of the infected blood, "was ja schon lange bekannt ist," is open to question, as previous workers, employing, it is true, other kinds of halteridia, have not been able to induce an infection by means of blood injections. In view of this fact, it is probable that the apparently successful inoculations with infected blood (and with mosquitoes) may be due at times to the lighting-up of a latent infection. (See also p. 240 and footnote p. 259.)

Although unconfirmed, except as mentioned above, still Schaudinn's views regarding the nature of the mosquito flagellates have been accepted by most zoologists. Some, indeed, have gone as far as to interpret the work done on the owl as applying to all trypanosomes, and in this they have gone beyond the position taken by Schaudinn himself; for he realized, as we know from his letters, that there were trypanosomes without an intracellular stage, and that there were halteridia which had no trypanosome stage. As a matter of fact, the whole question has narrowed itself down to the parasites of *Athene noctua* and their relation to the mosquito flagellates. It is not a question of a broad, general biological principle.

The work of the Sergents is of further interest, inasmuch as they found in the Malpighian tubes of mosquitoes (three out of 17) which fed on owls infected with *H. Ziemanni* the spirochetal forms described by Schaudinn. These flagellates, which they describe as being 25-30  $\mu$  long and 1  $\mu$  wide, are clearly different from the true spirochetes, which scarcely exceed 0.2  $\mu$  in width. Similar forms were not found in 52 *Culices* from the same brood as those used in the experiment; but whether these control mosquitoes were fed on non-infected birds is not stated. Repeated attempts to inoculate a barn owl, *Strix flammea*, either by injection of these flagellates or by the bites of mosquitoes, failed.

The measurements as given by the Sergents for *Sp. Ziemanni* agree perfectly with those of the long Herpetomonad form (*Tr. culicis*) which will be described later. This statement does not necessarily imply that the forms described by Schaudinn correspond to this trypanosome, for, as a matter of fact, his illustrations of *Sp. Ziemanni* (see his Fig. 17) show forms which have the blepharoplast posterior to the nucleus, and hence have a very long undulating membrane. Such is not the case with *Tr. culicis*. The forms mentioned, however, do resemble, remarkably, the free-swimming, long type of *Tr. avium*, as we have shown in the paper on "Bird Trypanosomes" (Plate 10). As to the possibility of the presence of true spirochetes see footnote p. 259.

Still more recently (1906) the Sergents have reported upon the presence of a *Her-*

*petomonas* in 13 out of 119 (11 per cent) *Culex pipiens* and in one *Stegomyia fasciata* which were raised from larvae in the laboratory. These mosquitoes, it should be noted, though fed either on infected owls or on clean canaries, showed as large a percentage of infections as was obtained by Schaudinn. They designate this organism as *Herpetomonas algeriense*, and describe two forms, motile and non-motile. The motile form was 12  $\mu$  long and 2.5  $\mu$  wide, and was provided with a whip which was 3.5–6  $\mu$  long. The large transverse centrosome was always posterior to the nucleus and about 0.56  $\mu$  from the broad rounded end. The non-motile forms were round, about 5.5  $\mu$  in diameter, and had a long, free flagellum measuring even 17  $\mu$  in length. Although the mosquitoes fed in part on owls infected with halteridia and *H. Ziemanni*, they do not mention finding the same trypanosomes as in the previous year. (See footnote p. 229.)

They also report on the presence of a very narrow flagellate in the digestive tube of the larvae of several *Anopheles* and of one *Culex*; and also of one nymph of *A. maculipennis*. The body averaged 16  $\mu$  in length and 3.5  $\mu$  in width, and was provided with a long free flagellum which measured 24  $\mu$ . They regard this organism as a *Herpetomonas* resembling the *H. jaculum* which Léger found in *Nepa cinerea*, and they suggest that possibly the latter obtains its infection by feeding upon the mosquito larvae. It will be seen from these observations of the Sergeants that they have described four kinds of flagellates in mosquitoes, two of which they identify with the forms of Schaudinn.

Summing up the investigations heretofore made regarding the flagellates of mosquitoes, it will be seen that two distinct types have been recognized, *Crithidia* and *Herpetomonas*, and that each of these is represented by one or more species. Exactly similar forms have been found in the digestive tract of other insects, some of which, it is worth noting, are not sanguivora.

The following list of the species described up to the present time may not be without interest:

<i>Crithidia fasciculata</i> , Léger, 1902 .....	In the gut of adult <i>Anopheles</i> and <i>Culex</i> ; also in larvae and pupae.
" <i>campanulata</i> , Léger, 1903 .....	In intestine near Malpighian tubes in larva of <i>Chironomus plumosus</i> .
" <i>minuta</i> , Léger, 1903 .....	In mid- and hind-gut of <i>Tabanus tergstinus</i> .
<i>Herpetomonas muscae-domesticae</i> , Burnett, 1851.	In the common house-fly; also in <i>Homalomyia scalaris</i> , <i>Pollenia rudis</i> , <i>Theicomysa fusca</i> (Léger).
" <i>Bütschlii</i> , Kent, 1881 .....	In intestine of <i>Trilobus gracilis</i> .
" <i>jaculum</i> , Léger, 1902 .....	In mid-gut of <i>Nepa cinerea</i> , adult and larvae.
" <i>gracilis</i> , Léger, 1903 .....	In Malpighian tubes of larvae of <i>Tanytus</i> .
" <i>Lesnei</i> , Léger, 1903 .....	In mid-gut near Malpighian tubes of <i>Dasyphora praetorum</i> .
" <i>subulata</i> , Léger, 1904 .....	In gut of <i>Tabanus glaucopsis</i> and <i>Hematopota italica</i> .
" <i>sarcophagae</i> , Prowazek, 1904 .....	In gut of meat-fly, <i>Sarcophaga hemorrhoidalis</i> .
" <i>bombycis</i> , Levaditi, 1905 .....	In butterfly of silkworm, <i>Bombyx mori</i> .
" <i>algeriense</i> , Edm. & Et. Sergeant, 1906	In gut of <i>Culex pipiens</i> and <i>Stegomyia fasciata</i> .
<i>Trypanosoma noctuae</i> , Schaudinn, 1904 .....	In gut of <i>Culex pipiens</i> as supposed stage of <i>Halteridium noctuae</i> .
" ( <i>Spirochaeta</i> ) <i>Ziemanni</i> , Schaudinn, 1904	In gut of <i>Culex pipiens</i> as supposed stage of <i>H. Ziemanni</i> .
" <i>Grayi</i> , Novy, 1906 .....	In gut of <i>Glossina palpalis</i> .
" <i>Tullochii</i> , Minchin, 1906 .....	" " " "
" <i>culicis</i> , N. Sp. ....	In gut of various culices.
" <i>Christophersi</i> , N. Sp. ....	In gut of dog-tick, <i>Rhipicephalus sanguineus</i> .

The unnamed herpetomonas forms observed in *Culex*, *Anopheles*, and *Stegomyia*, and also that met

with in *Stomoxys* by Gray, obviously cannot be included in the above list. This is also the case with a very small flagellate, one-fourth to one-fifth the diameter of a red blood cell, which Ziemann found in the ovaries of *Chrysops dimidiatus*, a biting fly in Camerun. It may be stated further that Rogers has recently designated the human parasite, *Piroplasma novovani*, as *Herpetomonas* of Kala-azar. The flagellate which Dutton and Todd found in two out of fourteen house mice in Senegambia, and which they compared with *H. Bäschlii*, is probably identical with *Tr. duttoni* which Thiroux later described in the mice of Senegal. It may further be stated that in *Nepa Léger* found a short oval flagellate about  $6\ \mu$  long, which he designated as *Otomonas tremula*.

The flagellates recently (1905) described in the gut of the sheep louse (*Melaphagus ovinus*) by E. Pfeiffer have been compared with *Crithidia* and *Herpetomonas*. They may be looked upon as "cultural" trypanosomes, as they show the blepharoplast anterior and close to the nucleus, while the undulating membrane is indicated with reasonable certainty in some of the illustrations given, especially in his Fig. 7. The intestinal epithelial cells were covered with rows of the resting forms, and, in addition, typical rosettes were observed. Unfortunately, no examination of the blood of the sheep from which the lice were taken is mentioned, and presumably was not made.

It is a well-recognized fact that *Tr. Lewisi* is carried from rat to rat by fleas and lice. The transmission by fleas was first established by Rabinowitsch and Kempner, although they were unable to detect the flagellates in the fleas by direct examination. In lice which have recently fed on rats infected with *Tr. Lewisi* this organism can be readily seen, and by placing freshly infected lice on a rat MacNeal was able to secure an infection. The fact that similar experiments failed, with lice containing more or less completely digested blood, goes to show that these insects do not play an active part as hosts, but are mere vectors or passive carriers of the parasites in much the same way as in the case of the tsetse fly.

According to the recent work of Prowazek (1905), the rat louse (*Haematopinus spinulosus*) is an intermediary host, inasmuch as he found trypanosomes, though in variable numbers, in almost all of the lice which fed on infected rats. The parasites were usually present in the stomach; twice they were seen in the Malpighian tubules, and even in the circulating blood, and once in an egg. That the flagellates were actively multiplying in the mid-gut was evident by the presence of divisional forms and of large rosettes or groups which covered the lower end of the stomach wall, especially in the region of the Malpighian tubules. The illustrations which he gives (such as Fig. 55) show undoubted "cultural" forms, since the blepharoplast is anterior to the nucleus and the flagella in the rosettes are directed centrally. Crithidia-like bodies with short flagella, as well as smaller involution forms, without whips, were found. The latter forms were either free in the gut or wedged deep between the cells. He described male and female forms, although, as in the case of *Herpetomonas muscae*, the difference between the two was not very marked.

Prowazek identifies these flagellates of the louse with *Tr. Lewisi*, but this is by no means proven, since he was unable to infect rats by placing upon them presumably infected lice. If the multiplying flagellates of the louse actually represent stages of the cultural form of *Tr. Lewisi*, it should manifestly be possible to produce an infection either by injecting the stomach contents of such lice after they had fully digested the blood which they had taken up, or by allowing them to feed upon clean normal rats. Our own experiments, as mentioned above, indicate that the transmission is effected by lice which have just fed on an infected rat and not by those which are void of fresh blood. While, therefore, it is quite reasonable to believe that the ingested trypanosomes multiply in the gut, it is clearly not proven. Although we have carefully

examined Prowazek's memoir, we have not been able to find any statement to show that lice taken from non-infected rats were free from these flagellates. Control examinations of this kind are as essential here as in the work upon mosquitoes. In the absence of such controls and of positive infection experiments, the possibility remains open that these organisms are mere intestinal flagellates of the louse in nowise derived from *Tr. Lewisi*.

In support of this view, and to illustrate the need of controls, we may cite some experiments in which mosquitoes were allowed to feed on rats infected with *Tr. Lewisi* and *Tr. Brucei*. In the stomach contents of these insects the original blood trypanosomes could be detected for 24 to 36 hours after the infective feed, as will be shown later. At times, *Crithidia* and *Herpetomonas* were present in these insects, and, were it not known that these belonged to the mosquito, they might well have been taken to represent multiplication forms of the blood trypanosomes.

As bearing upon the work of Prowazek should also be mentioned the observations of Nabarro and Greig, Gray and Tulloch, and of Koch regarding the presence of flagellates in the stomach of different species of tsetse flies. These organisms were regarded as multiplication forms of *Tr. gambiense* and *Tr. Brucei*, but the later studies of Minchin, Gray and Tulloch, and of one of us (Novy) show quite conclusively that such is not the case, and that these flagellates (*Tr. Grayi* and *Tr. Tullochii*) are mere harmless parasites of the intestinal tract analogous to those met with in mosquitoes. During the past year Koch himself has recognized the absence of any relation between these flagellates and *Tr. gambiense*. He now inclines to the belief that the tsetse flagellates are derived from the trypanosomes in crocodiles, on account of the similarity which they bear to the cultural forms of the latter.

Another instance of the presence of flagellates in insects is a recent observation made by Balfour (1906). While searching for a developmental cycle of *Hemogregarina Balfouri* in the flea (*Pulex cleopatrae*) which infects the Sudan jerboa, he found a flagellate which occurred in rosettes, or in slightly active forms, and as non-motile gregarines or amoebulae. He regards these organisms as being probably closely related to Léger's *Crithidia fasciculata*, and he further considers it likely that they are "merely parasites of the flea itself" and are unrelated to the hemogregarines of the jerboa. The fact of their presence in fleas fed on non-infected gerbils is important evidence on this point, and is in accord with the observations already noted.

It may further be said that Pricolo has found trypanosomes in fleas taken from mice (*Mus musculus*) which were infected presumably with *Tr. Duttoni*. The significance of the trypanosomes found in cattle and dog ticks will be discussed later.

While on a priori grounds one might be led to believe that the trypanosomes of vertebrates multiply in the digestive tract of insects, the fact remains that up to the present time no positive evidence has been brought forward in support of that view. There is, of course, no doubt as to the fact that trypanosomal infections are spread through the agency of insects, but these seemingly act the part of mere vectors and not of active hosts. It is probable that an exception to this statement is found in the case of kala-azar of India. The parasite of this disease, known as *Piroplasma donovani*, according to private communication from Captain Patton, develops in the gut of the common bedbug into flagellate forms, which then multiply and resemble the *Crithidia* of mosquitoes. This, it may be added, is apparently the only instance apart from the work of Schaudinn on the parasites of the owl, where an intracellular (leucocytic) parasite actually develops into a flagellate stage.



On the other hand, there is one group of organisms, the leeches, where the evidence as to the multiplication of trypanosomes in the gut is quite incontestable. The presence of flagellates in leeches (*Piscicola* and *Pontobdella*) was first noted by Leydig (1849) and later by Labbé in *Haemopsis sanguisuga* (15 days after removal from frogs), and it was on account of these observations that Doflein (1901) concluded that the fish trypanosomes were probably transmitted by means of these sanguivora.

Keysseltz was the first to approach the question methodically. Early in 1904 he showed that the leeches were to be considered as intermediate hosts, but the full results of his investigations were not published until 1906. He found a large percentage of leeches (*Piscicola geometra*), captured at large, to harbor in their stomachs variable quantities of flagellates, which because of their morphological characteristics he identified with trypanosomes and trypanoplasmes. Owing to the difficulty of obtaining leeches free from flagellates, he recognized the possible error in employing these in feeding experiments upon fish. He correctly reasoned that, though the fish flagellates would be thus introduced into the leech, it would be impossible to determine whether the multiplication forms observed belonged to them or were derived from the organisms already present in the leech. Hence, in order to obtain unquestionable results, it would be necessary to use leeches that had been raised from the egg and found, by control feeding experiments, to be actually free from flagellates. In his paper he describes, at length, the multiplication forms of trypanoplasmes, but the origin of these is not quite clear. He failed to produce an unquestionable infection by other injections of the stomach contents of the leech or by allowing the latter to feed upon the fish. It is probable that this failure was due to the use of non-susceptible species or such as had an acquired immunity. As will be shown below, Brumpt has succeeded in infecting fish, frogs, and eels by means of leeches.

Billet in 1904 also presented observations going to show that the *Tr. inopinatum* of the Algerian green frog multiplied very easily in the digestive tube of a leech (*Helobdella algira*). Like Keysseltz, however, he was unable to infect frogs either by injection of blood or by the use of infected leeches. Both results, it may be added, have been accomplished since then by Brumpt.

About the same time Léger experimented with loaches which harbor two kinds of flagellates, trypanosomes and trypanoplasmes. The intestinal contents of leeches (*Piscicola*), about four days after these had fed on loaches infected with *Tr. barbatulae*, showed numerous flagellates presenting the three types of Schaudinn. Similarly, leeches (*Hemiclepsis marginata*) placed on fish infected with *Trypanoplasma varium* showed in a few days numerous, small, almost filiform trypanoplasmes in addition to trypanosome-like forms. These observations confirmed the previous results of Brumpt, who noted the presence of prodigious quantities of trypanosomes, showing gregarine and herpetomonad forms, in *Hemiclepsis* taken from fish.

The several studies of Brumpt are of especial importance, as they clearly point to the rôle of leeches in the transmission of flagellates. He brought out the interesting fact that certain species of trypanosomes and trypanoplasmes develop in a given species of leech (*Hemiclepsis marginata*), but not in another (*Piscicola*), while others develop in the latter and fail to do so in the former. He made use of this fact in differentiating a large number of species of trypanosomes and trypanoplasmes. The flagellates found in the stomach and intestines present elongated, herpetomonad, or "cultural" forms, which are sufficiently characteristic to permit the recognition of

species. In these forms, as with most of our cultural forms, the blepharoplast is anterior to the nucleus.

According to Brumpt, the *Tr. granulosum* of the eel when sucked up by the *Hemodopsis* gives rise in a few hours, in the stomach, to pyriform or crithidia-like bodies, which then pass into the intestine, where they persist in the herpetomonad form for months. Hence he regards the latter as the ancestral form preceding the sanguicolous stage. Such leeches when placed on "clean" eels gave a positive infection in from four to six days. Contrary to Keysseltz, he was able to infect two carps, two chubs (chabots), and one cottus by placing upon these leeches taken from the same species of infected fish.

Similarly, Brumpt has shown that the *Tr. inopinatum* of the Algerian green frog when taken up by a small leech (*Ileobdella algira*) rapidly assumes the herpetomonad form, and that such leeches when placed on a frog, even as late as a month after the first feeding, produce a severe and even fatal trypanosomal infection after a period of incubation of from eight to 10 days. Unlike Billet, who, influenced by Schaudinn's work, was inclined to consider the leech trypanosome as a stage of the intracellular *Drepanidium*, he points out that there is no relation between these flagellates and the hemogregarines of frogs.

Lastly, it is in order to emphasize, as L  ger has done, the fragile distinctions which exist between *Crithidia*, *Herpetomonas*, and *Trypanosoma*. The first two genera are supposed to have no undulating membrane, and yet a rudimentary one is ascribed by L  ger to *H. subulata* and to *C. fasciculata*. On account of the proximity of the centrosome to the anterior end, it is evident that the undulating membrane must be but feebly developed, and hence cannot be readily seen. This is equally true of many of the cultural trypanosomes, and for that reason the failure to recognize the presence of an undulating membrane does not, or should not, exclude an organism from the trypanosome group.

Another difference which has been insisted on is the position of the centrosome or blepharoplast which, in *Crithidia* and *Herpetomonas*, usually lies by the side of, or anterior to, the nucleus, while in the blood trypanosomes it occupies a posterior position. This distinction, however, disappears when the cultural forms of trypanosomes are compared, as they properly should be, with these insect flagellates. Such comparisons are in order, for, as will be shown, the *Crithidia* and *Herpetomonas* present identically the same form in the test-tube as in the digestive canal of the insect. The native flagellates found in the gut of insects, therefore, are cultural forms, and as such are directly comparable with those of the blood trypanosomes. Now, in cultures of *Tr. Lewisii*, *Tr. Duttoni*, *Tr. rotatorium*, *Tr. Mesnili*, *Tr. Laverani*, etc., the blepharoplast occupies a position by the side of, or anterior to, the nucleus, and such forms might very properly be included under *Herpetomonas*. While this position of the blepharoplast is the usual one in cultural forms of trypanosomes, it is not always so. Thus, in some species, notably in the spirochete-like form of *Tr. avium*, it may lie posterior to the nucleus. This position, though rare, has also been observed in insect flagellates, namely *Tr. Tullochii* and *Herpetomonas algeriense*.

It is furthermore worthy of note, showing as it does the close relationship which exists between the cultural trypanosomes and the *Crithidia* and *Herpetomonas*, that all three types give rise to essentially the same gregarine forms. These are attached, whip foremost, to the epithelial cells of the intestinal wall, and are crowded together

in several layers; or they form large rosettes with central flagella or agglomerations filling the lumen of the tube. The polymorphism noted by Léger in the case of *Crithidia* and *Herpetomonas*, and by Schaudinn for *Tr. noctuae*, is equally marked in cultures of blood trypanosomes.

According to Léger, the main difference between *Crithidia* and *Herpetomonas* is in the large size of the adult monadian form of the latter as compared with that of the former. The existence of a double flagellum, as claimed by Prowazek for the *Herpetomonas* of the house-fly, if confirmed, would afford a better generic distinction. Gray, it is true, has found a double flagellum on the *Herpetomonas* of the *Stomoxys*, but it is by no means certain that this condition does not represent an early stage of division.

In view of the cultural characteristics of these three types, we believe it justifiable to consider them as belonging to the one genus *Trypanosoma*. The terms *Crithidia* and *Herpetomonas*, as in the case of *Proteosoma*, etc., will undoubtedly persist, but not as representing distinct genera. With the recognition of the trypanosomal nature, it does not follow that all of these flagellates are capable of growing in the blood of higher animals. Parasitism in the living blood must be looked upon as the result of a previous adaptation to the more or less digested blood in the intestinal tract of sanguivora. From this standpoint it will be seen that the ancestral, and for that matter the normal, type is the one met with in the digestive tube. Hence, the cultural forms, instead of being regarded as degenerations or involution types, in reality represent the normal phase, whereas the blood forms are transitory modifications due to environmental conditions.

#### METHODS EMPLOYED.

In order to study the flagellate infection of "wild" mosquitoes, the latter were captured in large numbers by means of Nocht's tubes. They were nearly all obtained from the thick undergrowth along the river bank in Ann Arbor; and, as soon as they were brought to the laboratory, they were placed in wire cages and allowed to feed on perfectly "clean" normal animals. By "clean" we mean animals which culturally and microscopically were shown to be free from trypanosomes and hemocytzoa. The mosquitoes were almost wholly of the genus *Culex*, and the most common species was *C. pipiens* or *pungens*. There were always a few *C. sylvestris* and *C. triseriatus*; the identification of these two we owe to Dr. L. O. Howard. The *Anopheles* were very scarce, only a few *A. maculipennis* and *A. punctipennis* being captured. For the percentage of infections see p. 242.

At first the insects were fed on young pigeons, but at times hawks, crows, rats, and guinea-pigs were made use of. In order to prevent the mosquitoes being killed, it is necessary, as a rule, to immobilize the animal. In the case of the pigeon this can be done by merely

tying the wings against the body. Hawks were managed best by placing them on a Latapie holder, but the crows were with difficulty prevented from eating up the mosquitoes. Contrary to the general statement that these insects will not feed on rats, we have found that they do so, though perhaps not so freely as on pigeons. On placing the neck of the rat in the pillory of a Latapie holder, the mosquitoes will feed undisturbed. In general, the guinea-pig is the animal of choice, for the reason that it is readily attacked by the mosquitoes, and, since it does not attempt to destroy them, there is no necessity to secure it in any way. Although by no means necessary, it is advisable to trim off some of the hair or feathers on the exposed animal.

Usually the exposure to the mosquitoes took place during the early part of the evening, but the number of insects which then feed is relatively small. By allowing the guinea-pig to remain in the cage all night, a much larger number will feed, especially during the early hours of the morning. Each cage contained a hundred or more mosquitoes, but the majority of these would not feed. After removing the animal from the cage, the insects which fed were taken up in Nocht's tubes and set aside in a cool, shaded place. The wide end of the tube was covered with netting, while the trap end held a plug of cotton, moistened with water. It is not advisable to moisten it with sugar-water unless the insects are to be kept alive for many days.

The mosquitoes which thus fed on normal blood were kept from 30 to 70 hours, when they were examined for flagellates. The time for making the examination depended somewhat upon the temperature, for in very warm weather complete digestion took place in about two and a half days, whereas when it was cool three or more days were needed. The percentage of findings was rather low when the mosquitoes were examined early—that is, when the stomach was still distended with undigested blood. Evidently the flagellates had not multiplied sufficiently to permeate the entire contents, and hence could be easily overlooked. Similarly, when the examination was delayed until complete digestion had taken place, or when sugar-water had been fed, the flagellates were equally difficult to find, owing to the fact that the free-swimming or monadian forms, as designated by Léger, had passed into the resting or gregarine stage. The best results were

always obtained when the examination was made toward the close of the digestion period—i. e., 40–60 hours after feeding.

In all of these experiments the mosquitoes were examined after a single feeding, and it is quite likely that the percentage of infections would have been even higher had they been fed two or three times so as to obtain an increased multiplication of the flagellates.

The mosquitoes were killed by inverting the tube over a filter paper on which was placed a drop of chloroform. This does not appreciably affect the flagellates, which remain alive and active in the stomach. After removing the thorax, wings, and legs, the digestive tube can be drawn out in the usual way, and the contents then forced out into sterile salt solution. Owing to the large number of examinations which had to be made, it was found that direct pressure on the dismembered body, by means of a sterile lance-shaped needle, gave about the same result, and this shorter procedure of getting at the stomach contents was the one chiefly used. By this means the fluid contents were expelled into a drop of sterile salt-citrate solution (a half per cent of each); the slide and cover-glass were previously flamed. The yellowish or whitish liquid was then examined with a No. 7 Leitz objective, and when necessary with an oil immersion lens.

When present, the flagellates are easily recognized by their more or less active motion. After a preliminary study of the organisms, the cover-glass was carefully drawn off, dried in the air, and stained by a modified Romanowsky method. When deemed advisable, the fluid remaining on the slide was taken up with a few drops of salt-citrate solution and either injected into birds or transferred to blood-agar tubes.

The flagellates present in the stomach contents were injected into 20 canaries, two mourning doves, and two blackbirds. The injections were made intrapleurally, and in no instance was it possible to observe the development of either trypanosomes or intracellular parasites in the blood of the birds thus inoculated. It may further be added that the pigeons, etc., after the mosquitoes fed on them were examined microscopically, and later cultures were made from their blood, but no evidence of infection was obtained.

The different cultures which were obtained were likewise injected into many animals such as sparrows, canaries, pigeons, doves,

hawks, owls, crows, rats, and mice. Inasmuch as such cultures were very rich in flagellates, the number injected was clearly many thousand times greater than would be introduced into an animal by the bite of a mosquito. The results were negative, as in the inoculations with the stomach contents. It may be that the failure to produce an infection was because of the use of non-susceptible animals. Some vertebrate may be capable of infection with the mosquito flagellates, but as yet nothing is known on that point.

One infection experiment is specially worth mentioning. Four sparrows which had been previously examined and found clean were injected with a second generation culture of Strain No. 85 (*Crithidia*). After eight days one of the sparrows showed halteridia, another showed halteridia and proteosoma, while the other two remained clean. Apparently, two out of four sparrows developed halteridia as the result of the injection and it will be noticed that the same percentage, three out of six, was obtained by the Sergeants in their work on the owl halteridium. It is reasonably certain, however, in our experiment that neither the halteridium nor the proteosoma was derived from the injected material. We have repeatedly found sparrows which at first were apparently clean, but after being kept in the laboratory for a week or two developed a halteridian or proteosomal infection. Latent infection is a common occurrence in sparrows, and for that matter in other birds, and, as pointed out on page 231, this condition may account for the apparently successful infections obtained by Schaudinn and by the Sergeants.

For the cultural work the blood agar was prepared in the usual way. Defibrinated rabbit's blood was employed, and this usually was added, in the proportion of one to two, to the melted agar, previously cooled to about 50°. The slanted tubes, when solidified, were inoculated, by means of a drawn-out tube pipette, with the citrate suspension of the stomach flagellates, after which they were capped and set aside at the room temperature.

The chief obstacle to the successful cultivation of the mosquito flagellates is the constant presence of bacteria in the intestinal canal of the insect. The bacteria, on account of their more rapid multiplication, may outgrow the flagellates from the very start, and thus render the medium unsuitable for the latter. This may be brought

about by a change in the reaction, or by the production of directly injurious products, or by the abstraction of oxygen. The abundant growth of aerobic bacteria may, as is well known, lessen the oxygen contents of a liquid to the extent of producing an anaerobic condition, which we know would interfere with the growth of trypanosomes in the culture-tube. In the stomach of the insect for some reason (possibly the reaction) the bacteria do not develop readily, and hence the trypanosomes are able to multiply, at times, to a very marked extent.

As might be expected, the flora of the insect gut is subject to considerable variation, and when slow-growing yeasts or bacteria predominate, the chances of getting the trypanosomes to grow, at least for one or two generations, are favorable. The conditions are seemingly more favorable when only a single species of bacterium or yeast is present. Our most successful results were obtained with such "pure mixed" cultures. The isolation of the trypanosomes from the accompanying bacteria or yeasts offers some difficulty, but this we were able to overcome, as will be shown later on, by plating on blood agar.

Cultures were attempted from 89 mosquitoes, all of which contained trypanosomes, some even in very large numbers. Of this number only seven were positive, Strains 10, 11, 18, 29, 69, 85, and A. Strains A and 69 were crowded out by bacteria after two and three days respectively. Strains 18 and 85 were kept alive for 20 days, through two and five generations respectively. Strain 11 was carried in 30 days through four, and Strain 29 in 166 days through 24 generations. Strain 10, which was started on August 5, 1905, is now (April 1907) in its 87th sub-culture.

Of the two cultures which were kept up one, Strain 10, was *Crithidia fasciculata* and the other, Strain 29, was a *Herpetomonas*, *Tr. culicis*. The former was associated with a yeast and the latter with a minute bacillus. The growth was very rapid and extremely rich, so much so that in warm weather it was necessary to check it by keeping them in a cold room. These two cultures will be described in their proper place.

#### CRITHIDIA FASCICULATA.

This flagellate is fairly common in the gut of ordinary "wild" mosquitoes of the genus *Culex*, but it is not the only organism that

may be present. Thus, out of 882 mosquitoes examined in 1905, flagellate infection was met with in 126 (14.3 per cent). Of this number, 55 had apparently only *Crithidia* (6.2 per cent), while 52 had only *Herpetomonas* (6.0 per cent), and 19 had both parasites (2.1 per cent). During the summer of 1906 the percentage of infected mosquitoes was about a third of that found in the preceding year. Thus, of 746 mosquitoes 37 were infected (4.95 per cent). Of this number 20 had only *Crithidia*, 16 only *Herpetomonas*, and one had a double infection. It will be seen from this that infection with the former is slightly more common than with the latter. The relatively small number of infections obtained last year was probably due to the cutting-away of most of the undergrowth along the river.

The existence of a mixed infection with these two flagellates has not heretofore been recognized, and as a result, the two types, when occurring together, have been described as different stages of one and the same organism. This is clearly the case with the *Herpetomonas subulata* of Léger, for the small pear-shaped, truncated forms which he shows (Ref. 35, p. 614, Figs. 4 and 5) are *Crithidia*, while the longer ones (Figs. 1, 2, and 3) are true herpetomonads. Again, the *Crithidia minuta* which Léger, on account of the presence of the long form (Ref. 35, Fig. 6, p. 614; also Ref. 33, Fig. 3, p. 185) later placed in the genus *Herpetomonas*, is without doubt a true *Crithidia*, since it shows the short pear-shaped as well as the elongated, cylindrical form which belongs to this type. These two figures of Léger should be compared with Fig. 6, Plate 8. Our reason for this belief is based upon the results of the cultivation of the two types, for it will be shown that in cultures the *Crithidia* and *Herpetomonas* present entirely distinct forms and retain their individual characteristics through numerous sub-cultures. In cultures of *Crithidia* the typical long herpetomonad form, gradually tapering posteriorly, is never found, and similarly in cultures of the *Herpetomonas* there is an entire absence of the peculiar short crithidian type.

On account of the facts just stated we cannot agree with Lühe in his characterization of the genus *Crithidia*. According to Prowazek, the *Herpetomonas* of the house-fly has a double flagellum arising from a terminal blepharoplast, but this observation, as already pointed out, needs confirmation. The absence of the double flagellum,



the position of the centrosome at some distance from the anterior end, and the presence of a feebly developed undulating membrane are regarded by Lühe as the most important generic features of *Crithidia*. These properties, however, are based presumably upon the long form of *H. subulata*, which is entirely different from that of true *Crithidia*. Hence, until it is definitely shown that the *Herpetomonas* of the house-fly always possesses a double flagellum, it will be well to retain this designation for similar forms found in other insects.

*The genus Crithidia, if it is to be retained, is characterized by peculiar, short, oval or pyriform bodies which are usually rounded or obtuse posteriorly, while the anterior end is truncated, or even slightly depressed, and bears a short, straight flagellum. In this short form the nucleus is near the posterior end, and the blepharoplast is by its side. Somewhat longer cylindrical forms, tapering very slightly, or rounded at each end, may be present, and these are provided with a long flagellum. In this form the nucleus is near the center, and the centrosome is usually about midway between the nucleus and the anterior end. Both forms may occur in rosettes with flagella directed centrally. The undulating membrane and the posterior diplosome, as seen in the Herpetomonas, are absent.*

Some variation in the form and size of the *Crithidia* is met with in the body of the mosquito. This must obviously be the case in view of the changes in the composition and concentration of the intestinal contents. It will be sufficient to point out the great difference in the rate of digestion of the blood in the stomachs of mosquitoes according as they are kept at a high or a low temperature. Furthermore, the ingestion of plain water or of sugar-water is an additional factor in bringing about greater or less alteration in form and size. In the cultures on blood agar these differences are readily observed, as will be presently shown. The environmental conditions are essentially the determining cause of the two types of Léger—the free or monadian, and the fixed or gregarine-like, resting stage.

The possibility of the existence of several species of *Crithidia* is obvious, and it is likely that their differentiation, as in the case of bird trypanosomes, can be effected more readily by the cultivation method than by mere morphological study. In the course of this investigation we have grown five strains of *Crithidia*—Nos. 10, 11,

69, 85 and A; but unfortunately all but the first were soon lost. It was noticeable, however, that Strain No. 85 differed in some respects from the others, but this variation might have been due to changes in the medium caused by the different kinds of bacteria present. What has been said here with reference to the existence of several species of *Crithidia* is also true for the *Herpetomonas*. The question is worth investigating in localities where flagellate infection of mosquitoes is very common. Taken as whole, the *Crithidia* which we have studied, in mosquitoes and in cultures, agree so well with the description of *C. jasciculata* which Léger found in *Anopheles* that we have identified all of our forms with this species.

#### MOSQUITO FORMS.

The flagellates may be present in the stomach contents in varying numbers. In some instances but one or two may be found on the slide, while in others they are exceedingly numerous. They may be single or in large masses which fill the field of a No. 7 objective. For convenience the *Crithidia* will be divided into the short and the long forms.

As seen in the living condition the *Crithidia* usually appear as short, thick, oblong cells. They are more or less actively motile and travel about with the whip foremost. The body of the smaller forms is about  $3-4\mu$  long and  $2\mu$  wide, while the larger ones measure  $6-8\mu$  in length by about  $3\mu$  in width. The contents are colorless and apparently homogeneous.

The short forms are often found grouped in rosettes with the whips inside. These groups may consist of large numbers of cells, just as in the case of the rosettes of *Tr. Lewisi*, *Tr. avium*, etc. The short form, furthermore, may become attached by means of the flagellum to the intestinal wall, more especially to the lower end of the Malpighian tubules, and as a result the entire wall may be covered with radiating masses consisting of countless numbers of parasites. A similar arrangement of the cells is to be seen about minute globules of air (aerotropism), and this behavior so characteristic of cultural trypanosomes will be discussed later on.

The most important features are brought out in the stained preparations. The thick wide cells may be said to be pear- or cigar-

shaped (Plate 7) and have been compared by Léger to barley grains. The posterior end is either round or slightly pointed. The body of the cell may, or may not, taper toward the anterior end, which is either square or slightly concave. Round forms, from 4 to 6  $\mu$  in diameter, may also be present. The relatively large round nucleus lies near the posterior end in the short forms and at about the middle in the longer ones; close to the nucleus in front or on the side is the centrosome. From the achromic zone surrounding the latter, the flagellum can be seen to pass forward and out *at the middle* of the anterior end. The free portion of the whip is short and straight, or but slightly bent. The flagellum is more difficult to stain than in the case of other trypanosomes, and hence the cells will often appear to be devoid of whips. A colorless channel, marking the site of the flagellum, can be seen in such specimens, extending from the blepharoplast to the anterior end (Figs. 1 and 2, Plate 8). In the stained preparations the body of the cells measures from 3 to 8  $\mu$  in length and from 1.5 to 3  $\mu$  in width. As the free whip is from 2 to 5  $\mu$  long, the total length of the cell may be put down at from 5 to 14  $\mu$ . These measurements, it may be said, have been made on preparations made from mosquitoes which contained no herpetomonad forms. The absence of very long forms should be noted.

#### CULTURAL FORMS.

As mentioned on p. 241, one of the crithidian strains which were isolated from mosquitoes (No. 10) has been cultivated now for 20 months, and during that time has passed through 87 generations or sub-cultures. For more than a year it has been grown as a strictly pure culture.

On the ordinary blood-agar medium it grows even more rapidly than any of our bird trypanosomes, so much so that during the warm weather it is necessary to restrain the growth by placing the tubes in a cool room. Without this precaution it would be necessary to transplant the culture every three or four days. The growth is extremely rich, and glairy patches or colonies are readily made out. Obviously, depending on the changing conditions of the medium and temperature, some variation in form and size will occur, but on the

whole the cultural features of our strain have been maintained unimpaired during these many months.<sup>1</sup>

It is important to note that these cultural forms are very often of the same size and shape as are those found in the mosquito. This fact will be readily seen on comparison of the Plates 7, 8, and 9. As in the gut, so in the tube we can distinguish between a very short and a moderately long form. The short thick cells (body 4 to 8  $\mu$  long and 2 to 3  $\mu$  long) with truncated anterior are identical with like forms seen *in vivo*, the gregarine forms of Léger. On the whole, the conditions in the test-tube, notably the abundant food-supply and absence of bacteria and other inhibiting influences, are more favorable than in the insect, and there is a greater tendency to develop the long or monadial form. This is entirely different from that of the *Herpetomonas*, and, as will be seen, it is much shorter than the latter. The body of the long form measures from 8 to 15  $\mu$  in length and from 1.5 to 2  $\mu$  in width. The free flagellum may be as long as the body, and hence the total length of the cells may range from 13 to 26  $\mu$ . The width of some of the forms, especially just before division, may reach 4.5  $\mu$ . These long cultural forms are represented by similiar ones (10-15  $\mu$  in length), resembling *Herpetomonas*, which are found at times in the mosquito. The cylindrical or cigar-shape, the obtuse posterior end, and the very short taper to the anterior end of body, together with the absence of the posterior diplosome, makes it possible to distinguish the elongated *Criithidia* from the true *Herpetomonas*. (See p. 254.)

It is important to note that the long cultural forms, at times, show a marked constriction near the posterior end (Figs. 4 and 5, Plate 9). Although we have not ascertained the real significance of this constriction, we are inclined to consider it as a process preparatory to actual division. This feature corresponds, in a remarkable way, with the constriction of the oökinetes of *Tr. noctuae* as described by Schaudinn (see his Figs. 1, 2, and 3).

While this fact, together with the rounded posterior end, goes to establish a certain identity between *Tr. noctuae* and *Criithidia*, it may be well to point out that the former does not agree in all respects

<sup>1</sup> At the present time the vegetative power of this organism is such that it can be grown on ordinary inclined agar without the addition of blood. In several attempts to secure "blood" forms, by resorting to cultivation in collodium sacs in rats, no growths were obtained.

with the latter. This is especially true of the anterior end, which in *Tr. noctuae* is prolonged along the base of the flagellum and clearly shows an undulating membrane. These two characteristics, and the presence of posterior diplosomes, agree with the structure of *Herpetomonas*. It would seem therefore as if Schaudinn's *Tr. noctuae* was a composite of these two flagellate forms.

Rosette formation is a common feature of the cultures and corresponds to the characteristic grouping met with in insects. The smaller groups of from two to 10 cells usually consist of short thick ovals or spherical bodies which, as in the case of the rosettes of *Tr. avium*, *Tr. Lewisi*, etc., show little or no motion. At times the rosettes consist of many hundreds of individuals, which may be partly or wholly of the elongated type, and in such cases the latter show an active swaying motion. The flagella are directed centrally as in the case of all multiplication rosettes.

The rosettes are easily distinguished from ordinary agglutinations. The latter result in irregular masses, consisting at times of numerous cells, all of which have the flagella on the outside. The beginning of agglutination may be seen when two cells become adherent by their posterior ends; or side to side with the flagella extending in opposite directions.

Another very important feature which seemingly is shared by all cultural trypanosomes, but not by the blood forms, is the phenomenon of *aerotropism*. Thus, if a minute bubble of air is introduced under the cover-glass, the *Crithidia* will range themselves in compact rows, two to five deep, around the globule. Every cell has its whip directed toward the air-bubble. This behavior of *Crithidia*, and of cultural trypanosomes in general, to air corresponds, as will be seen, to the so-called gregarine or resting stage observed in the insect. In the latter the flagellates are attached in the same manner to the epithelial cells, and it is reasonable to believe that this so-called resting stage is in reality an aerotropic manifestation due to the alteration and absorption of the stomach contents. Certain it is that this resting form when detached from the epithelium, by pressure or currents of liquid, is at once able to move about quite freely.

The cultural fluid, in addition to the rosettes, shows many free, actively moving, darting forms. These may travel through the field

with great rapidity and in nearly straight lines. Most of the single forms, however, for some reason become attached by means of their flagella to the glass surface and are unable to do more than sway or wriggle about the attached point.

The length of the flagella varies with that of the cell, and in general is about as long as the body proper. In the small forms the whip is short and stiff, while in the elongated type it is long and flexible. In the former it is not unusual to see the flagellum retracted to a short stiff bristle as a result of the passage of the blepharoplast from the anterior end of the cell to the side of the nucleus which lies at or near the further end. (See Figs. 1, 2, and 3, Plate 9.)

The larger *Crithidia* show a distinct yellowish green color, which may be due to staining with blood pigment. The contents of the cell appear homogeneous, or show at most a very fine granulation. In heavily stained preparations deeply colored granules can be seen filling the cell (Plates 8 and 9). This stippling is at times difficult to bring out. In Strain 85 the cultural cells were totally colorless and showed very minute but clear, distinct globules.

The presence of an undulating membrane has not been satisfactorily established. The short, oblong forms, on account of the square anterior end and median insertion of the flagellum, are clearly devoid of an undulating membrane. In the very longest forms the anterior end is not carried along the flagellum, as in the case of *Herpetomonas* and most trypanosomes, but is seen to terminate more or less abruptly. Such long forms, when examined in the living condition, especially when the cell has become sluggish, give evidence of the presence of an undulating membrane at the base of the flagellum. The periplast is apparently carried beyond the obtuse or truncated end of the cell as a short triangular membrane which shows a *distinct wave motion*. With the retraction of the flagellum, as in the short form, all evidence of this structure disappears. In the stained preparations it has not been possible to demonstrate this rudimentary membrane, although indications of it are to be seen in Fig. 6, Plate 8.

The presence of an undulating membrane, imperfect as it is, definitely places the *Crithidia* among the *trypanosomes*, and this position is further indicated by the obvious analogies which this type presents in cultures to those of the latter.

The very small rounded forms, especially when the flagellum does not stain, resemble very much, as Léger first pointed out, the *Piroplasma*. The resemblance to the peculiar parasite of kala-azar, *Piroplasma donovani*, is even more marked. The development of the latter into flagellate forms in the test-tube (Rogers), and in the bedbug (Patton) suggested to the former a relationship to *Herpetomonas*, while the latter is of the opinion that this parasite is a *Crithidia*.

Divisional forms, of course, are very common in the cultures, and hence such material presents the very best opportunity for the study of the life-history of the parasite. The forms met with are the same as those seen in the mosquito. The short, wide ovals divide longitudinally into two equal halves, each of which shows the typical square anterior end (Fig. 3, Plate 8).

As mentioned on p. 239, the inoculations of birds and mammals with large amounts of the flagellates, obtained from tube and flask cultures, failed to produce an infection. Hence, so far as we know, the *Crithidia* is a parasite peculiar to the mosquito, and perhaps to other insects.

#### TRYPANOSOMA (HERPETOMONAS) CULICIS, N. SP.

It has already been shown that the ordinary "wild" mosquitoes of the genus *Culex* may harbor either *Crithidia* or *Herpetomonas*, or both kinds of parasites at the same time. From the figures given on p. 242 it will be seen that the infection with *Herpetomonas* is nearly as common as that with *Crithidia*.

The *Herpetomonas* which we have studied resembles in general the long monadian form of *H. subulata* which Léger found in *Tabanus glaucopis*, and at first we were inclined to identify it with the latter. Léger's form, however, is distinctly longer, the body on an average being  $30\mu$  long by  $1.5-2.0\mu$  wide, the flagellum being  $20-25\mu$  long. Moreover, as pointed out on p. 242, the description of this species undoubtedly includes a true *Crithidia*. The *Herpetomonas algeriense* which the Sergents described, shortly after the appearance of our preliminary note, was found in 13 specimens of *Culex pipiens* and in one of *Stegomyia fasciata*, and was characterized as having the centrosome in the motile forms, posterior to the nucleus, and also by a rounded resting form having very long flagellum. The unnamed type

which they found in the digestive tube of a larva of *Anopheles maculipennis* resembles our form more closely than does the preceding. It appears that, notwithstanding its frequent occurrence, this mosquito flagellate has not been heretofore recognized, or at all events, it has not been differentiated from the accompanying *Crithidia*. We therefore consider it as a new species and propose to designate it as *Trypanosoma culicis*.

While this organism has been found only in the several *Culices* examined by us, it is quite probable that it will be recognized in other mosquitoes, including *Anopheles* and *Stegomyia*. It is hardly necessary to add that a further investigation of the mosquito flagellates is needed in order to pass upon the question of the multiplicity of species both of *Crithidia* and *Herpetomonas*. The wide geographic distribution of these two types is seen in the fact that Patton has found both of them to be very common in the *Culex fatigans* at Madras. The probable presence of the two types or genera in other insects is evident from Léger's work on the Tabanides.

According to the recent studies of Prowazek, the herpetomonads of the common house-fly and of the meat-fly (*H. muscae domesticae* and *H. sarcophagae*) possess a double flagellum which terminates in a minute diplosome, and this, in turn, connects with a single large blepharoplast by means of two rods or rhizoplasts. As pointed out, if these observations are confirmed, it will be necessary to restrict the term *Herpetomonas* to those organisms having the same flagellar arrangement as in the type species *H. muscae domesticae*. It seems to us, however, somewhat premature to insist, as Lühe has done, upon the limitation of this genus to these two double flagellar forms. The herpetomonadian forms have been noted in diverse insects, but in none has this flagellar structure been seen. Such an arrangement is certainly absent in the herpetomonadian forms which we have studied, namely *Tr. culicis* and *Tr. Grayi*.

On the other hand, there are reasons for believing that the double flagellar arrangement described by Prowazek represents an early divisional stage. Such undoubtedly is the case with the forms described in the *Stomoxys* by Gray. It is to be remembered that the first evidence of division is usually seen in the enlargement of the blepharoplast, and then in the formation of a new flagellum (see



Fig. 3, Plate 11). As a matter of fact, in rapid multiplication, such as is seen in the segmenting forms of *Tr. Lewisi*, the cultural forms of *Tr. Laverani* (Ref. 46, Fig. 6, Plate 6), etc., the new whip appears before the blepharoplast shows any appreciable sign of division. The existence of a double flagellum would effectually remove the flagellates of the house-fly from the trypanosomes. The *Herpetomonas* of the mosquito, which it will be seen is a true "cultural" trypanosome, shows considerable similarity to *H. muscae*.

#### MOSQUITO FORMS.

What has been said on p. 244 with reference to the variation in the form and size of *Crithidia*, and the possible existence of several species, holds equally true for the *Herpetomonas*. The conditions which obtain in any two insects are not necessarily alike, and, for that reason, considerable difference in size and form may be expected without recourse to the assumption that very long forms represent one species and very short ones another. The variation in size is an important argument in support of the view that the intestinal flagellates are true cultural types.

In the living condition the *Herpetomonas* is very active and moves about rapidly; so much so that it is often difficult to follow it from field to field. It moves with the long flagellum foremost in exactly the same manner as do the cultural trypanosomes, *Tr. Lewisi*, *Tr. avium*, etc. The contents of the cell appear colorless and homogeneous.

On account of the rapid motion the form is at times difficult to make out, but in cells which have slowed down, and especially in stained preparations, it will be seen that the cell is a narrow spindle tapering gradually toward each end. The presence of an undulating membrane is clearly indicated in the longer forms.

The herpetomonad forms seen in the living preparations vary considerably in length. In a few mosquitoes very long forms, the body measuring from 30 to 35  $\mu$ , have been observed. In these the flagellates were all of this size, and, had the cultivation attempts been successful, it would have been possible, perhaps, to determine whether this long form was a distinct species. In the majority of the mosquitoes the herpetomonad forms were from 12

to 20  $\mu$  long. The width of the living cell is more uniform and varies from 0.5 to 1.0  $\mu$ . Hence, the appearance of the long, slender form is not unlike that of the spirochete-like stage of *Tr. avium*. It is a mistake, however, to compare either of these types with the true spirochetes, inasmuch as there is an entire absence of the spiral winding so characteristic of the latter and, moreover, on staining, there is brought out the usual trypanosomal structure which is wholly lacking in the real spirochetes (*Sp. Obermeieri*, *Sp. Duttoni*, etc.).

The presence of these very long, slender forms in the mosquito, however, is very strongly suggestive of Schaudinn's *Spirochaeta Ziemanni*. It will be remembered that Schaudinn himself practically acknowledged that this organism was not a true spirochete, since it had the ordinary trypanosomal structure. In view of this similarity, it may well be asked whether or not some of the forms of this *Herpetomonas* correspond with the flagellate stage of *Hemoproteus Ziemanni* as described by Schaudinn. Certain it is that the long, slender forms correspond to the size (25 by 1.0  $\mu$ ) given by the Sergeants for the flagellate stage of *H. Ziemanni*. On the other hand, it should be noted that Schaudinn's Fig. 17 shows a long undulating membrane, due to the position of the blepharoplast which is posterior to the nucleus, as in the cultural form of *Tr. avium*. For the cultivation of spirochetes from owls see p. 259.

"In addition to the long and short forms mentioned, various stages of involution are met with as in the case of ordinary cultures. The more or less spherical forms with long flagella, kite-shaped bodies and spindles, rounding up posteriorly, correspond to the resting stage of Léger.

Divisional forms are often seen in the living preparations. The dividing cells are shorter and wider than the ordinary free-swimming forms. The division is longitudinal and is fairly equal. Multiplication rosettes with centrally directed whips are occasionally found, but they are by no means so common as in the culture tube. Agglutination groups or rosettes have also been observed, and it is important to note that in these groups the flagella are directed outward as in the case of *Sp. Ziemanni*.

The herpetomonad forms found in the stained preparations

made from mosquitoes vary greatly in length, from 10 to 45  $\mu$ . They may for convenience, if for no other reason, be divided into four types: (1) long; (2) medium; (3) short; and (4) wide.

1. In the long spindles the posterior two-thirds of the body gradually tapers to a fine point, while the anterior third is drawn out along the base of the flagellum so that it is difficult to tell just where it ends (Fig. 2, Plate 10). The length of the body in these long forms varies from 25 to 35  $\mu$ , and the greatest width is from 1.0 to 1.5  $\mu$ . The free whip measures from 5 to 10  $\mu$ , so that the total length ranges from 30 to 45  $\mu$ . *With respect to size, as pointed out above, this type corresponds to the forms which the Sergeants identified with the flagellate stage of H. Ziemanni.* . (See p. 231.)

2. The cells of medium size,<sup>1</sup> those from 15 to 25  $\mu$  in length (not including the whip), likewise taper toward both ends. The width may be a trifle greater than that given for the preceding type—that is, from 1.5 to 2.5  $\mu$  (Figs. 1, 2, 3, Plate 10). *This type corresponds in size to the indifferent forms of Tr. noctuac as given by the Sergeants.*

3. In the short form the body measures from 7 to 15  $\mu$ , and the whip is from 3 to 8  $\mu$  long. The width is about the same as that of the preceding. These short forms also show the characteristic tapering ends, though not to the same extent as in the long types (Fig. 6, Plate 10). *They may be compared with the male forms of Tr. noctuac as given by the Sergeants* (p. 227).

4. In addition to the three types given above, which are by far the most common, there is another present, in mosquitoes and in cultures, though it is much less frequent. This is a wide, fairly long form in which the characteristic tapering is less pronounced (Figs. 5 and 6, Plate 12). The body measures about 20  $\mu$  in length and about 2.5–3  $\mu$  in width. The free whip is short, about 5–8  $\mu$ . This type stains a deeper blue than any of the above, and the contents show granules as well as colorless globules. *This form may be identified with the female form of Tr. noctuac as described by the Sergeants* (see p. 227). It recalls the broad or “female” type of *Tr. Lewisi* as met with in the blood of rats, and may well be compared with the broad or “female” type of *Tr. Grayi* (Ref. 45, Plate 15).

<sup>1</sup> The long and median narrow forms described above are suggestive of the slender or “male” type of *Tr. Grayi*, as will be seen on comparison of Figs. 1 and 2, Plate 10, with Figs. 1 and 2, Plate 15 (Ref. 45).

Inasmuch as all four of these types have been studied in the cultures of *Tr. culicis* as well as in the mosquito, there is every reason to regard them as belonging to one species. Similar variation in form and size, it may be added, is common among other cultural trypanosomes, for example, *Tr. avium*.

The nucleus in the narrow forms is compact and cylindrical, and as wide as the cell (compare *Tr. Grayi*, Ref. 45, Figs. 1, 2, and Plate 15). In the "female" type two nuclei may be present without there being any evidence of multiplication (Fig. 5, Plate 12). Similar polynuclear forms have been described in the cultural forms of *Tr. Grayi*, *Tr. Lewisi*, etc.

The blepharoplast is round or oval and measures  $0.5-0.7\ \mu$ . It lies either immediately in front of the nucleus or about midway between the latter and the anterior end. We have never seen it lie posterior to the nucleus as in *H. algeriense*. Starting from the blepharoplast, a single flagellum passes forward along the side of body and is finally prolonged as the free whip. In the long forms the evidence of the presence of an undulating membrane is unmistakable (Fig. 2, Plate 10).

By far the most characteristic feature is the presence of a *posterior diplosome* or *acrosome* similar to that which we first described in the cultural form of *Tr. Laverani* (Ref. 46, p. 290, Plate 6), and in *Tr. Grayi* (Ref. 45, p. 407, Plate 16). This body is present in all four types as well as in the dividing forms. It appears to be a definite, constant structure, as much so as the nucleus or the blepharoplast. The presence of this diplosome has not been observed in *Crithidia*.

As a rule, the diplosome stains less readily than does the nucleus, and this behavior is probably due to the protecting action of the surrounding matter. In the very thin, or in crushed, cells this structure is as sharply defined as the blepharoplast (Fig. 6, Plate 11). The diplosome is usually rod-shaped, about  $0.4$  by  $1.6\ \mu$ , with a more or less evident median constriction, in which case it presents the appearance of a diplococcus or diplo-bacillus (Fig. 1, Plate 10). It is nearly as large as the blepharoplast.

The diplosome apparently divides before any divisional change is seen in the nucleus or blepharoplast (Figs. 4 and 5, Plate 10). As

the result of such division two distinct rods or two diplococcus-like bodies form. We have never seen more than two diplosomes in a cell, although special search was made. A suggestion of three such bodies may be seen in Fig. 5, Plate 10. In the first three types the diplosome is always posterior to the nucleus; in the fourth or "female" type it may lie in the anterior part of the cell.

A somewhat similar structure, derived from the blepharoplast, has been described by Schaudinn in connection with the development of the oökinete into the female and male forms of *Tr. noctuae* (see his Figs. 2 and 4). In that case the blepharoplast is said to give rise by three consecutive divisions to eight small, compact nuclei, each of which in turn divides, forming a smaller nuclear body. In the female cell, these eight pairs of nuclear bodies disappear, whereas in the male oökinete they become the nuclei and blepharoplasts of eight male trypanosomes. In his study of the *Herpetomonas*, Pro-wazek has likewise noted a posterior diplosome which is apparently in relation to the blepharoplast.

The diplosome which we have described is present in all forms of *Tr. culicis*, as found in the mosquito as well as in those grown in the culture-tube. The similar bodies of Schaudinn, it should be said were noted only in the oökinete. Just what the significance of the diplosome may be it is impossible to state, but our observations go to show that it is a distinct morphological structure and is not directly derived from the nucleus or blepharoplast. As pointed out above, the diplosome presents the earliest evidence of a coming division of the cell. As the latter divides, each half is equipped with a diplosome (Figs. 2 and 6, Plate 11), as in the case of *Tr. Laverani*.

The direct division of the long, slender forms of *Tr. culicis* have not been observed. They apparently shorten and widen just before division takes place. At all events, the common dividing form, as found in the mosquito and in the culture, is an oval body about  $3\mu$  wide and  $8-10\mu$  long. The acrosome divides first; then the blepharoplast, which lies just in front of the nucleus, divides and gives rise to a new flagellum, after which the nucleus divides (Fig. 3, Plate 11). The division is equal and longitudinal, and hence the resulting two cells are of about the same size about  $8-10\mu$  long and  $1.0\mu$  wide (Fig. 6, Plate 11).

The female type apparently divides longitudinally without becoming round or oval (Fig. 6, Plate 12). The two large nuclei which result from division separate, and one lies in the anterior and the other in the posterior part of the cell. Hence, one of the cells resulting from the division will have its nucleus at or very near the posterior end. In this dividing form the diplosomes lie either between or in front of the nuclei (Fig. 5, Plate 12), as in the case of *Tr. Grayi*.

*Involutions*.—In some mosquitoes the *Herpetomonas* loses its typical form, rounds up posteriorly, and becomes first club-shaped, and finally more or less spherical. This change likewise occurs in the culture-tube, and the resulting bodies correspond to the round form seen in cultures of *Tr. Lewisi*, *Tr. avium*, etc. Such bodies, measuring about  $5\mu$  in diameter or 5 by  $8\mu$ , represent in part the resting or gregarine form of Léger and others, and should probably be interpreted as degenerations or involutions, since the plasma in these altered cells does not stain as well as in normal cells. In such cells, owing to the feebly stained protoplasm, the nucleus, blepharoplast, and diplosome stand out very prominently (Figs. 3, and 4, Plate 12). As opposed to the view that these are degenerations is the fact that these forms show the various division changes. Thus, one may find all stages from the cell with a single nucleus, blepharoplast, diplosome, and whip to one in which each of these structures is doubled.

#### CULTURAL FORMS.

Of the seven cultures of mosquito flagellates only one (Strain No. 29) gave a good growth of *Herpetomonas* which was successfully carried through 24 generations or sub-cultures in the course of five months, from August 6, 1905, to January 12, 1906. Another, Strain No. 18, went through two passages, but was then lost on account of overgrowth by the accompanying bacteria. The cultures made from Mosquitoes 10 and 11 which had a double infection showed some herpetomonad as well as crithidian forms (in the first generation), but for some reason the former disappeared on transplantation, and the second and subsequent generations showed only *Crithidia*. The disappearance of *Herpetomonas* from these cultures indicates some

unfavorable action either of the particular kind of bacteria which were present or of the *Crithidia*.

Strain No. 29 was associated with a minute diplo-bacillus which seemingly exerted no injurious action as long as the cultures were transplanted regularly, once or twice a week. When the transplantation was delayed for a longer interval, most of the trypanosomes, as is usually the case, became degenerated, and hence the sub-cultures then made were poor in flagellates and relatively rich in bacteria. Otherwise there was no reason why this culture could not have been kept up almost indefinitely as in the case of *Crithidia*.

The isolation of a strictly pure culture was more difficult than with *Crithidia* on account of the adherence of the minute bacillus to the sticky surface of the flagellate. Several times, however, almost pure cultures were obtained from colonies on blood-agar plates, and, without doubt, this method on further replating would have given a strictly pure growth. Unfortunately, just at this time the culture was lost through an oversight in not making regular transplants. The streak cultures on rabbit blood agar, in a Petri dish, gave moist, glairy colonies of almost pure trypanosomes. The colonies varied in size from mere points up to that of a large pin-head.

The forms met with in the culture-tube, it may be said, correspond in every respect to those observed in the mosquitoes. The four types found *in vivo* are also present *in vitro*, and in size and form the latter are not to be distinguished from the former. This fact accords with the observations on the crithidian cultures, and confirms the view that the flagellates of mosquitoes and other insects represent cultural forms.

Nothing is known as yet regarding the forms which these flagellates would assume if they were able to grow in the blood current. It is probable, however, that they would give rise to large typical trypanosomes corresponding to the blood forms of *Tr. avium*, *Tr. Lewisi*, etc. It is evident, therefore, that the cultural forms, instead of being degenerations and involutions as suggested by some, really represent the ancestral primitive type from which the peculiar blood forms have evolved as a result of adaptation to the living fluids of the body. From the cultural stand-point the "drawn" blood does not

correspond to the living blood, by which term we may designate that which is circulating in the body.

The cultures of *Tr. culicis* show small forms, the body of which measures but 6 or 7  $\mu$ . These may have a tapering posterior end, or one which is blunt and rounded. They are readily distinguished from the wider and truncated *Crithidia*. Small, rounded or oval forms, 4 or 5  $\mu$  in diameter, are also present, and such usually have a very long flagellum, 12–16  $\mu$  in length. The most common form is that of the typical *Herpetomonas* with slender, tapering posterior end. These are from about 12 to 15  $\mu$  in length, and from 1 to 1.5  $\mu$  in width. Occasionally longer forms up to 35–40  $\mu$ , and the “female” forms already mentioned, are met with (Plate 12).

Rosettes, with the flagella directed centrally, are present in the cultures, but not as commonly as in the case of *Crithidia*. Agglutination of two or more cells is a common condition. The two cells may be attached either by their posterior ends or lengthwise, side by side, with the flagella pointing in opposite directions. When agglutinated in large masses the cells are also grouped with the flagella on the periphery. As with other flagellates the presence of soft debris or fat globules favors the formation of large agglutinations.

Acrotropism is as pronounced as in the case of *Crithidia* and other cultural trypanosomes. The long, slender herpetomonads arrange themselves around the globule in dense layers, side by side, with the flagella inward, and thus present a very striking object.

The slender forms travel rapidly, with the whip foremost, and while in motion appear as mere lines. The body is straight or slightly curved, and is rather stiff—a condition especially noticeable when the motion has decreased. The contents of the living cell have a greenish tint and homogeneous, but on careful examination very fine granules can be seen, especially in the posterior half. The presence of a slight, but distinct, undulating membrane can be observed in the slowly moving, or arrested long forms. Short, wide dividing forms (6–8  $\mu$  long by 4–5  $\mu$  wide) corresponding to those observed in the mosquito are fairly common (Plate 11).

Inoculations of the cultures were made into a variety of birds and mammals, as stated on p. 239, but in no case was it possible to secure an infection showing either trypanosomes or cytozoa. While it is



possible that this flagellate has a blood stage in some vertebrate, there is as yet no evidence of that condition, and, for the present at least, the *Tr. culicis* must be regarded as a parasite peculiar to the mosquito.

# SPIROCHETES.

The so-called *Spirochaeta Ziemanni* which Schaudinn described in *Culex pipiens*, as the flagellate stage of *Haemoproteus Ziemanni*, is clearly a trypanosome and not a true spirochete. In view of this fact, it is of interest to note that Töpfer, according to Mühlens and Hartmann, has succeeded in cultivating a spirochete from the owl. Although Töpfer has not published as yet his results, we are assured by Dr. Schilling that the organism mentioned is a real spirochete, and, that being the case, it throws much light upon the exact nature of "*Spirochaeta*" *Ziemanni*, which Schaudinn himself practically acknowledged to be a trypanosome. Töpfer's observation would go to show the existence of a spirochete infection of owls similar to that known in chickens and geese, bats, etc. It is possible that mosquitoes feeding on owls, thus infected, would show true spirochetes in the digestive tube.\*

\* Since the above was written one of us (Novy), through the very great courtesy of Dr. Töpfer, has been able to examine a preparation and a photograph of the spirochete which he has cultivated from the blood of an owl infected with *H. Ziemanni*. There is no question as to the spirochetal nature of the organism which Dr. Töpfer has isolated. It is an interesting and important fact that this organism presents in cultures not only typical spirochetes 10-15  $\mu$  in length, but also smaller forms consisting of but one or two turns. The latter correspond to the S-, or vibrio, forms of the cholera spirillum. The cultures also show minute round bodies which contain one or two chromatin granules and these bodies doubtless represent the resting stage of *Sp. Ziemanni* as described by Schaudinn. They may be compared with the well-known involution forms of the cholera vibrio, and their presence, together with the short forms mentioned above, affords good evidence of the bacterial nature of this spirochete.

Dr. Töpfer's organism in all probability corresponds to the *Spirochaeta Ziemanni* of Schaudinn. The long spirochetes, and the short forms representing the minute ones found by Schaudinn in the gut of mosquitoes (the result of the rapid multiplication of the indifferent form), and also the round or resting forms, are in accord with Schaudinn's description. It appears probable, therefore, that the *Spirochaeta Ziemanni* after all is a spirochete and not a trypanosome. But assuming that this identity is established it does not go to prove that the spirochete in question is a stage in the development of the intracellular parasite *H. Ziemanni*. On the contrary, it indicates the existence of a spirochetal disease of owls corresponding to that of geese and chickens. Indeed, Schaudinn himself noted that the owls, inoculated with suspensions of mosquitoes, showed an enormous increase of indifferent spirochetes very much like that seen in infections with *Sp. anserinum*. He considered this as the acute stage which was later followed by the appearance of the sexual forms of *H. Ziemanni*. It may be said, as a result of our own observations, than an infection with *Sp. Obermeieri* or *Sp. Duttoni* seems to exert a marked favoring action upon the multiplication of trypanosomes and probably of other protozoa, and hence the change from one type to the other, as noted by Schaudinn, is suggestive of a double infection. It will be seen, therefore, that the owls are subject to infection with at least four parasites, namely *H. Ziemanni*, *Halleridium*, *Trypanosoma*, and *Spirochetes*. This fact of a multiple infection in owls together with the normal presence of flagellates in mosquitoes goes to show the great difficulties under which Schau-

In view of the frequent occurrence of spirochetes in the mouth, stomach, and intestines of higher animals, the presence of these organisms might be expected in the gut of insects. As a matter of fact, several instances of this kind are now known. The three pathogenic spirilla, *Sp. gallinarum*, *Sp. Obermeieri*, and *Sp. Duttoni*, have been found in their insect hosts. The Sergeants in 1906, reported the finding of spirochetes in large numbers in a preparation made in 1901, from the digestive tube of a larva of *Anopheles maculipennis*. These measured 8-17  $\mu$  (average 13  $\mu$ ), and showed from 1.5 to 4 weak, spiral turns. At times they were agglutinated. A very similar, if not identical, organism, *Spirillum glossinae*, was described shortly afterward by Novy and Knapp. It was present in the stomach contents of two tsetse flies (*Glossina palpalis*). The single cell or short form with three to four turns measured 8  $\mu$ , while the double cells were about 15  $\mu$  long. Another instance of this kind is given by Wenyon,<sup>1</sup> who found spirochetes in lice which infested mice having *Sp. muris*. There was no sign of multiplication, and attempts at infecting mice by means of lice were unsuccessful.

In our own work with mosquitoes we have not been successful in finding real spirochetes, although special search was made for them. In the stained preparations made from several mosquitoes we did find spirochete-like forms but the presence of foreign particles made their nature rather uncertain. We learn through a personal communication from Captain Patton that true spirochetes are very common in the mosquitoes about Madras. It is evident, therefore, that in some localities the genuine spirochetes may be detected in mosquitoes, flies, etc., more readily than in others.

#### BEHAVIOR OF MAMMALIAN TRYPANOSOMES IN MOSQUITOES.

As bearing upon the question of the multiplication of trypanosomes in the gut of insects, it may be of interest to present some results obtained with *Tr. Lewisi* and *Tr. Brucei*. Wild mosquitoes were allowed to bite infected rats and guinea-pigs, and after a variable

*dinn worked while endeavoring to establish the life-cycle of H. Ziemanni.* Now that attention is called to the probable existence of a spirochetal disease of owls it is important that a special effort be made to detect the organism in their blood and to demonstrate the action of pure cultures upon clean birds, and their behavior in the gut of mosquitoes.

<sup>1</sup> *Jour. Hyg.*, 1906, 6, p. 583.

length of time the stomach contents were examined and injected into rats and mice. The combined contents of from two to ten mosquitoes were injected into each animal.

No multiplication of these trypanosomes was observed in the stomach even in mosquitoes which were re-fed. The number of trypanosomes ingested rapidly decreased, and dead forms were found after a few hours. In the Lewis mosquitoes, flagellates were found alive as late as 65 and even 114 hours, but these did not resemble either the blood or the cultural forms of *Tr. Lewisi*, and without doubt were ordinary herpetomonads. Unquestionable survivals were noted occasionally as late as 36 and even 48 hours. The *Tr. Brucei* died out more rapidly than the rat trypanosomes, but a few were seen to persist in some mosquitoes as late as 30 and even 36 hours.

Of 16 rats injected with the Lewis mosquitoes, six died from bacterial infection in from three to 11 days, and 10 were under observation for over a month. In none of these rats were trypanosomes detected. The injections were made one and one-half, three, six, nine, and twelve hours after the infective feed.

Of 10 mice inoculated with the Bruce mosquitoes, two developed an infection after an incubation period of eight days. One of these, which received the stomach contents of two mosquitoes, nine hours after feeding, died in 11 days; the other, which received the contents of five mosquitoes 12 hours after feeding, died in 10 days. Two mice which received stomach contents, 30 and 36 hours after feeding, died in four and five days respectively from bacterial infection. Of four rats inoculated with the stomach contents only one became infected after a period of incubation of nine days. This received the stomach contents of three mosquitoes, 14 hours after feeding, and died in 13 days. On looking over our laboratory notes we are unable to find any record of successful infection of mice with mosquitoes which had fed 24 to 36 hours before. Hence the statement to that effect, made in a previous paper, is erroneous. Positive results, it will be seen, were obtained at the end of nine, 12, and 14 hours, but not always. On account of variable conditions in different mosquitoes, the infection experiments do not always succeed in the stated intervals or even in shorter ones. Thus, we obtained failures at the

end of six, 12 and 14 hours, as well as in 18, 24, 30, 36, and 42 hours.

It will be seen from the above that the conditions in the stomach of the mosquito are vastly more unfavorable than in the culture-tube. In our previous studies on *Tr. Brucei*<sup>1</sup> it was shown that the sluggish survivals in the culture-tube (after inoculation with fresh blood) were usually non-virulent after the fourth or fifth day, and that if infection did occur it was only after a long period of incubation. In the above mosquito experiments the survivals failed to infect after 14 hours. The failure to infect with *Tr. Lewisi* is probably due to the same cause. Apropos of these tests, it may be recalled that Bruce was unable to infect dogs by injecting the stomach contents of tsetse flies after an interval of more than half an hour from the time of the infective feed. The fact that the ingested trypanosomes lose their virulence so rapidly in the stomachs of insects indicates a loss of functional activity, especially the power of multiplication and hence such insects cannot play the part of an active host. In view of these unfavorable conditions, it is difficult to see how the mammalian trypanosomes which are no longer able to multiply, even when placed in susceptible animals, could have a life-cycle within such a host.

#### TRYPANOSOMES OF TICKS.

The detection of trypanosomes by Weber, in the blood of some cattle suffering with Texas fever, and the subsequent finding by Schaudinn of like organisms in restrained preparations of the intestinal contents of ticks, taken from infected cattle in Finland, led the latter to set up the working hypothesis that the development of the piroplasmes of cattle and dogs is similar to that of the halteridia. That is to say, Schaudinn was led to believe that the piroplasmes possessed a trypanosomal stage resembling that which he had described for the intracellular parasites of the owl.

In view of the position assumed by him, and its acceptance by some writers, it will be of interest to note that trypanosomes have been recently found at Madras in a dog-tick, *Rhipicephalus sanguineus*, by Captain S. R. Christophers, I. M. S. He found the organisms in only one specimen of the tick, and the slide containing these flagel-

<sup>1</sup> *Jour. Infect. Dis.*, 1904, 1, p. 17.

lates he very kindly placed at our disposal. The preparation, which was made from the lower end of the Malpighian tubule or portion of the rectum, showed a considerable number of well-stained trypanosomes. We propose to designate this flagellate as *Tr. Christophersi*. Another instance of flagellate infection of a tick is mentioned by Doflein, who quotes Leydig as finding them in *Ixodes testudinis*.

From the photographic reproductions given in Plate 13 it will be seen that the flagellates of the dog-tick are typical "cultural" trypanosomes. They are considerably larger than the *Crithidia* and *Herpetomonas* of mosquitoes, and are quite different from the trypanosomes in tsetse flies, as will be seen on comparison with the microphotographs of the latter.<sup>1</sup> They resemble somewhat the cultural forms of certain bird trypanosomes which we have very briefly described under Type 4 in a previous paper,<sup>2</sup> but this of course does not imply that they are derived from avian flagellates. It is more likely that they will be found to come from mammalian forms.

The *Tr. Christophersi* is characterized by a prominent undulating membrane which extends over about half the length of the body. The roundish nucleus lies at about the middle of the cell, and is nearly of the same width as the latter. The small blepharoplast, about  $0.7\ \mu$  in diameter, is usually close to and on one side of the nucleus, but at times it is found to lie immediately in front. From the widest central portion the body tapers in both directions. Anteriorly it gradually merges with the flagellum, and posteriorly it terminates in a sharp, pointed end very much as in the case of *Tr. Lewisi*. Minute colorless globules are present, especially in the posterior half of the cell. Division is longitudinal and unequal.

The trypanosomes are fairly uniform in size and are all of the same type. The total length is from 30 to  $45\ \mu$ . The body is usually about  $25\ \mu$  long, but may vary from 20 to  $35\ \mu$ . The width is from  $2.5$  to  $4\ \mu$ . The length of the free flagellum is  $8-12\ \mu$ .

The size of these tick flagellates is of itself sufficient to exclude any possibility of their being developmental stages of the *Piroplasma canis*. If there was any doubt on this point, it has definitely been set aside by the recent studies of Captain Christophers, from which

<sup>1</sup> *Jour. Infect. Dis.*, 1906, 3, Plates 15, 16, 17.

<sup>2</sup> *Ibid.*, 1905, 2, p. 292.

it appears that he has succeeded in following out the complete life-history of this organism. His work demonstrates that in the life-cycle of this cytozoon there is no trypanosomal stage. There is therefore very little probability of a flagellate stage being found in the other piroplasmæ, cattle, horse, etc. All of the known facts with reference to insect trypanosomes go to show that those present in ticks are either derived from like forms in the blood of animals on which they feed or that they are parasites peculiar to these insects.

#### RELATION OF THE INSECT FLAGELLATES TO THE BLOOD TRYPANOSOMES.

This subject has been discussed (p. 234), in part, in connection with the account of the various investigations on insect parasites. It has been shown that abundant caution must be used in the interpretation of such findings. A natural parasitism of the intestinal tract is clearly a widespread and common condition, and it must be conceded that up to the present time no incontestable evidence has been produced to show that the sanguicolous trypanosomes are able to grow and multiply in insects. The facts, such as they are, indicate that the biting insects merely transmit the surviving unaltered trypanosomes which they have taken up with the blood, and that such transmission can occur only during the few hours following the infective feed. The innocuousness of biting insects, such as *Glossina morsitans*, *Gl. palpalis*, rat lice, etc., after they have digested the infected blood, can be interpreted in only one way, namely, that the ingested trypanosomes have died out or are about to do so.

The conditions in the gut of insects are comparable with, but hardly as favorable as, those which are offered in the culture-tube. Knowing the difficulties encountered in the cultivation of *Tr. Brucei*, *Tr. gambiense*, etc., it will be readily understood that even greater ones must exist in the intestinal tract. Hence it is that surviving forms of these organisms are rarely found in the gut after 24 to 36 hours following their introduction with the blood. The more rapid the digestion and absorption of the food, the more rapidly will such organisms perish in the digestive tube.

In the case of *Tr. Lewisi*, *Tr. avium*, etc., which may be looked upon as nearly "saprophytic" on account of the extreme ease with

which they can be grown in the test-tube, it is evident that they are more likely to take on the "cultural" form, and thus adapt themselves to the new conditions than are the truly pathogenic trypanosomes. For this reason it is conceivable that these trypanosomes may multiply in the gut of lice, mosquitoes, etc., but the proof is as yet wanting.

The only instance which we have of the multiplication of the blood trypanosomes in the gut of sanguivora is that afforded by the leeches. The large quantity of blood taken up, the anti-coagulative action of the secretions, the slow digestion, and the low temperature are factors which enable trypanosomes of the type mentioned above to multiply in this intestinal tract, and thus, so to speak, secure a foothold. Hence a leech, once infected, is capable of transmitting the infection to eels, fish, and frogs, etc., even after some weeks have elapsed from the time of the infective feed.

It has been repeatedly shown that the flagellates present in the intestines of insects and leeches present the characteristics of cultural forms of trypanosomes, and this fact is now definitely proven for *Crithidia* and *Herpetomonas*. These organisms do grow and thrive in the test-tube vastly better than in the mosquito, and the rich cultures thus obtained afford the best material for the study of "the parasite's life-history and biology." The *ex cathedra* condemnation by Woodcock of the cultivation method on the ground "that this is not a zoological method of research" will hardly be accepted by the true investigator who is not bound down by set forms and procedures. Scientific progress demands the application of new methods, and though "the trypanosomes are not bacteria," it is safe to say that the cultivation method as applied to these organisms will yield results which might be utilized to advantage by the morphologist.

Bearing in mind the fact that the blood trypanosomes when grown *in vitro* lose their original form and present cultural characteristics which ally them to the gut flagellates, it will be seen that the blood form in all probability represents the latest adaptation, to a new environment, of organisms which originally are derived from the intestine of insects. The adaptation to living matter is obviously the sequel of a previous existence on dead matter, and for that reason the intestinal or cultural forms instead of being involutions or degenera-

tions, closely represent, as Brumpt first pointed out, the ancestral or primitive type of the sanguicolous trypanosomes.

#### RELATION OF INSECT FLAGELLATES TO CYTOZOA.

A brief statement of this question, by way of a summary, will not be out of place in connection with the foregoing topic. The demonstration of at least two distinct flagellates, *Crithidia* and *Herpetomonas*, in all stages of ordinary mosquitoes, goes to show beyond any doubt that these forms bear no immediate phylogenetic relation to the intracellular parasites. The recognition of this fact more than ever invalidates the observations of Schaudinn on halteridium and leucocytozoon. It is imperative that his work should be repeated with the same species of bird (*Athene noctua*), the same species of mosquito (*Culex pipiens*), and if possible in the same locality, namely Rovigno, and that under experimental conditions which will leave no room for criticism. The several possible sources of error have already been pointed out (p. 230), and until that work is confirmed it is unwise to give it undue prominence and to base on it far-reaching conclusions as some zoologists have done.

We are firmly of the belief that the large typical trypanosome which is commonly present in owls corresponds closely to the large form or "female" of *Tr. avium* which we have described and illustrated in a previous paper.<sup>1</sup> The similarity will be evident on comparing our photographs with the one given by Nocht<sup>2</sup> and designated as the female form of *Spirochaeta Ziemanni*. Hence our further belief that the owl trypanosome is no more related to the *H. Ziemanni* than is *Tr. paddae* to the halteridium which often accompanies it. Our work on the "Bird Trypanosomes" has shown conclusively that the blood trypanosomes are definite species and are not stages in the life-history of cytozoa. In our next paper we expect to show beyond doubt that *H. Ziemanni*, in the blood, has a different development from that worked out by Schaudinn. Moreover, we have allowed "raised" mosquitoes to feed on hawks infected with *H. Ziemanni*, and have not been able to secure the "spirochetes" or other evidence of flagellate stages. It may be added, parenthetically,

<sup>1</sup> *Jour. Infect. Dis.*, 1905, 2, Plate 3.

<sup>2</sup> Kolle and Wassermann's *Handbuch*, Ergänzungsband, Erstes Heft, 1906, Plate 3, Fig. 3.



that we have not been able to detect flagellates in a large number of larvae which were examined for that purpose, and hence this may account for the negative results just mentioned. Had we allowed "wild" mosquitoes to feed on the infected hawks, we should have obtained, without doubt, a flagellate infection as did Schaudinn and the Sergeants. The fact that the latter used "raised" mosquitoes does not exclude the presence of intestinal flagellates, since it has been shown that in the warm countries infection of larvae is a not uncommon occurrence. The present study shows that the ordinary "wild" mosquitoes do harbor herpetomonad forms which conform fully to the "spirochetes" which the Sergeants considered identical with those described by Schaudinn (p. 253). The relation of Töpfer's owl spirochete to *H. Ziemanni* has been discussed on p. 259.

The position of *Trypanosoma noctuae* is essentially like that of *Spirochaeta Ziemanni*. Inasmuch as the halteridium infection of owls and hawks in Michigan is much less frequent than that with the leucocytozoon, we have not been able to make any feeding experiments with mosquitoes in this type of infection. The study of the natural mosquito parasites, however, shows the same possible source of error and leads to the conclusion that the so-called *Tr. noctuae*, instead of being a developmental form of the intracellular halteridium, is a common inhabitant of the gut of mosquitoes, and is probably a mixture of crithidian and herpetomonad forms.

It is certainly significant that, in spite of the stimulus which Schaudinn's observations have given to the examination for flagellate stages, no one has yet demonstrated such stages for any one of the intracellular parasites. Thus, Billet's supposition that the *Tr. inopinatum*, in frogs, was the flagellate stage of a *Drepanidium* has been effectually disproved by Brumpt. The guarded supposition of Koch that the trypanosome present in crocodiles is in relation either with the accompanying hemogregarine or with the tsetse-fly flagellates (*Tr. Grayi*) will hardly stand the test of actual experiment. Lastly, the work of Thiroux has clearly shown the absence of any genetic relation between *Halteridium* and *Tr. paddae*, and the recent studies of Christophers are equally conclusive as to the absence of a flagellate stage in the life-cycle of *Piroplasma canis*. The "flagellate-like"

forms recently described by Kinoshita for this organism have very little that is suggestive of trypanosomes.

#### ISOLATION OF TRYPANOSOMES FROM MIXED CULTURES.

F. G. NOVY AND R. E. KNAPP.

In general, it may be said that bacteria once introduced into a culture of trypanosomes tend to outgrow and check the development of the flagellates. It is probable that this injurious action is due largely to the production of poisonous products or to an alteration in the reaction of the medium. The antagonism between certain bacteria and these flagellates is deserving of attention. In exceptional instances, however, the bacteria exert little or no interference, and may even be apparently beneficial. While in the former instance the trypanosomes soon die out, in the latter the mixed culture can be kept, by frequent transplantation, almost indefinitely. We have, for example, maintained a mixed culture of *Tr. Mesnili* associated with a coccus for nearly a year. Similarly, *Critidia jusciculata*, which was isolated from a mosquito along with a yeast, was cultivated in this mixed form for nearly six months. Likewise, the *Tr. culicis* was grown in mixed culture with a minute diplo-bacillus for nearly five months. In these instances the foreign organisms did not alter, or but very slowly, the color of the blood agar, and it would seem from other trials as if a rapid destruction of hemoglobin by the accompanying bacteria was an injurious factor.

The isolation of trypanosomes from the gut of insects in a pure condition is hardly to be expected, owing to presence of various bacteria and yeasts. There is a possibility, however, of obtaining a "pure, mixed" culture—that is, where the flagellate is associated with only one kind of foreign organism—and such cultures as seen from the above examples are more favorable for transplantation than when a number of different species are present. The need of strictly pure cultures is soon recognized when it is desired to study the pathogenic action of the trypanosomes.

We have attempted to secure a separation by three different methods. In the first method fairly good results were obtained by inoculating the culture medium some 2 or 3 cm. above the condensation fluid, and then keeping the tubes in an almost horizontal position.

This was done with the object of allowing the motile trypanosomes to travel away from the accompanying bacteria or yeasts, and thus reach the fluid at the bottom of the tube. Several times almost pure cultures were obtained in this way, especially in the case of the *Criithidia* which was associated with a large yeast. Fairly good results were also obtained with the *Herpetomonas*, but in that instance the minute bacillus was more readily carried by the sticky flagellates into the condensation fluid. In the presence of motile or slimy bacteria this method fails entirely.

In the second method the attempt was made to utilize the marked aerotropism of the trypanosomes. As has been pointed out, the cultural flagellates tend to gather in compact layers about minute bubbles of air. On removing such air-globules by means of a capillary it would seem as if almost pure transplants could be obtained. The method, however, was not tested sufficiently to show its practicability.

The method which gave perfectly satisfactory results was as follows: By means of a glass spatula, made by drawing out the end of a glass rod, a little of the mixed culture was spread in a series of streaks over six Petri dishes containing solidified blood agar. Ordinary agar may be used in the first three dishes, since the desired dilution is not attained until in the last three. The form of Petri plate known as the "Kriegsministeriums-Modell," made by Greiner and Friedrichs, is particularly adapted for this purpose, inasmuch as it can be effectively sealed by means of a wide rubber band. The sealed dishes were then set aside at room temperature for 10 or 12 days. The last plate or two of the series was found to contain isolated colonies of trypanosomes, which could then be transplanted in the usual way to the test-tube. By this means we were able to secure a pure culture of *Criithidia*, and were almost successful with *Herpetomonas*. Without doubt this method will be found useful in future studies of the flagellates found in the gut of insects and other sanguivora.

#### SUMMARY.

The results and conclusions of this study can be recapitulated as follows:

1. Of the "wild" mosquitoes examined by us approximately 15 per cent in one year, and about 5 per cent in that following, were

found to be infected with flagellates belonging to two easily differentiated types, *Crithidia* and *Herpetomonas*. The percentage of infections is influenced largely by local and seasonal conditions.

2. By the cultivation method it has been possible to isolate two of these flagellates, namely *Crithidia fasciculata* and *Tr. culicis*; and it has been shown that the plate method is applicable for the separation of trypanosomes from the accompanying bacteria and yeasts.

3. The organisms obtained *in vitro* correspond to those observed *in vivo*, and hence the intestinal types represent true cultural forms.

4. The two types are common in other insects, and, instead of being classed as distinct genera, they should be placed under the trypanosomes.

5. The mosquito flagellates are not stages of intracellular organisms, but are probably parasites peculiar to the insects.

6. The inoculation of available experimental animals with the *Crithidia* and *Herpetomonas* has given negative results.

7. The *Tr. noctuae*, as described by Schaudinn, presents the mixed characteristics of *Crithidia* and *Herpetomonas*, while that of the Sergents agrees with the *Herpetomonas*; as such, it does not represent a stage of the *Halteridium*.

8. The cultivation by Töpfer of a spirochete from the blood of an owl, presumably identical with Schaudinn's *Sp. Ziemanni*, indicates the existence of a new spirillosis. The forms which the Sergents identified with *Sp. Ziemanni* were probably not spirochetes but trypanosomes, more especially the long form of a *Herpetomonas*. The conclusion seems justified that neither the flagellates and spirochetes found in mosquitoes nor the trypanosomes and spirochetes found in the blood of owls are related to the leucocytozoon.

9. The trypanosomes which are at times present in ticks are not developmental forms of *Piroplasma*. It has already been shown that the trypanosomes in tsetse flies are not related to *Tr. gambiense* or *Tr. Brucei*.

10. The possibility of the trypanosomes of birds and other vertebrates developing in the gut of insects, while not excluded, has not been demonstrated. In the mosquito, *Tr. Lewisi* and *Tr. Brucei* lose their infectiousness, more or less rapidly, but the enfeebled organism may survive in the gut for 36-48 hours. The conditions

in the digestive tube of insects is not as favorable as in the test-tube, and this fact goes to show that insect carriers, such as tsetses, are mere passive hosts.

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## EXPLANATION OF PLATES.

The accompanying photographs are all taken at the uniform magnification of 1,500 diameters, and, as such, they are directly comparable with those of other cultural trypanosomes previously given. The preparations were stained by a modified Romanowsky method.

## PLATE 7. CRITHIDIA FASCICULATA.

(As found in mosquitoes.)

FIG. 1.—*Crithidia* from mosquito No. 99.

FIG. 2.—Same preparation, showing *Crithidia* and *Herpetomonas*. Note the square anterior end and the short flagellum of the former; the diplosome posterior to the nucleus in the latter.

FIG. 3.—Elongated *Crithidia* from the same preparation.

FIG. 4.—*Crithidia* from mosquito No. 56.

FIG. 5.—*Crithidia* from mosquito No. 46.

FIG. 6.—*Crithidia* from a culture nine days old (Gen. 1); compare Figs. 5 and 6; the former is derived from a mosquito, the latter from a culture. Note the similarity in size and structure, showing that the wild *Crithidia* are really cultural forms *in vivo*. Compare also with cultural forms shown on Plates 2 and 3.

## PLATE 8. CRITHIDIA FASCICULATA.

(Cultural forms of Strain No. 10.)

FIGS. 1 AND 2.—Multiplication rosettes, showing large and small cells. Note the unstained channel in some showing position of whips which are directed centrally; also the small round or oval blepharoplast adjoining the nucleus. Nine-day culture, Gen. 1.

FIG. 3.—Dividing form, final stage; showing two nuclei, two blepharoplasts, and two flagella, one of which is longer than that of the parent cell. Three-day culture, Gen. 39.

FIG. 4.—Part of a rosette of elongated *Crithidia* with flagella directed centrally; note the stippling, Gen. 32.

FIG. 5.—Group of short *Crithidia* from the same preparation as Figs. 1 and 2. Note the truncated anterior end.

FIG. 6.—Elongated *Crithidia* from the same preparation as preceding.

## PLATE 9. CRITHIDIA FASCICULATA.

(Cultural forms from Strain No. 10. Figs 1, 2, and 3 are from a preparation of the 20th generation or sub-culture.)

FIG. 1.—An agglutination group of *Crithidia*.

FIG. 2.—Short oval form showing square anterior end, blepharoplast adjoining the nucleus, and a short flagellum, bent within the cell.



FIG. 3.—Short, thick form with concave anterior end; blepharoplast and flagellum as before.

FIG. 4.—Goblet-shaped *Crithidia*. Note the square anterior end and the constriction posterior to the nucleus. Also the dark granules at posterior end. Compare with the constricted forms shown in Schaudinn's. Figs. 1*b*, 2*b*, and 4*b*. From a third generation, two days old.

FIG. 5.—Normal and goblet-shaped *Crithidia* from the same preparation as preceding.

PLATE 10. TRYPANOSOMA (*Herpetomonas*) CULICIS.

(As found in mosquitoes; compare with cultural forms on Plate 12.)

FIG. 1.—Pale, slender form of medium length; showing flagellum, blepharoplast, nucleus, and posterior diplosome or acrosome. From mosquito No. 52. Compare with cultural form shown in Fig. 1, Plate 12.

FIG. 2.—Longer form from same preparation; showing undulating membrane flagellum, blepharoplast, nucleus, and posterior body. Compare with cultural form shown in Fig. 2, Plate 12.

FIG. 3.—From same preparation as preceding. Note the short new flagellum (?) indicating initial division.

FIG. 4.—A medium form, showing two diplosomes or pairs of posterior bodies. From mosquito No. 99.

FIG. 5.—A similar cell from mosquito No. 52, showing two diplosomes, and possibly a third against the nucleus.

FIG. 6.—Small form (male ?) from same preparation as preceding.

PLATE 11. TRYPANOSOMA (*Herpetomonas*) CULICIS.

(As found in mosquitoes and in cultures.)

FIG. 1.—Rounded-up form from mosquito No. 4. Note the two large diplosomes between the blepharoplast and nucleus.

FIG. 2.—Short divisional form, showing two flagella, two blepharoplasts, two nuclei, and two diplosomes or pairs of posterior bodies. From mosquito No. 52.

FIG. 3.—Cultural form from Strain No. 29, Generation 17, showing early stage of division. Note the thickening of the base of flagellum which is due to the presence of a short new whip; division of the blepharoplast; undivided nucleus and the presence of two diplosomes or pairs of posterior bodies.

FIG. 4.—Divisional form from same preparation as preceding; showing two flagella and two nuclei with rather indistinct posterior bodies. Two blepharoplasts are present, but one lies partly over the other.

FIG. 5.—Divisional form from same preparation, showing two prominent blepharoplasts as well as two flagella and two nuclei.

FIG. 6.—Divisional form from same preparation, showing complete division; prominent posterior bodies and blepharoplasts.

PLATE 12. TRYPANOSOMA (*Herpetomonas*) CULICIS.

(Cultural forms from the 17th generation of Strain No. 29.)

FIG. 1.—Pale, slender form, corresponding to that shown in Fig. 1, Plate 10. Note the prominent diplosome.

FIG. 2.—Longer form, showing undulating membrane and posterior diplosome. Compare with the mosquito form shown in Fig. 2, Plate 10.

FIG. 3.—Rounded-up form, showing flagellum, blepharoplast, nucleus, and prominent diplosome. Compare with Fig. 1, Plate 11.

FIG. 4.—Very short and long male and indifferent forms; also a rounded-up cell as in preceding figure, but showing two diplosomes or pairs of posterior bodies.

FIG. 5.—Large and rather rare form (female ?) with two nuclei. The diplosomes are in the anterior portion.

FIG. 6.—Large (female ?) form, similar to preceding, in process of division.

PLATE 13. *TRYPANOSOMA CHRISTOPHERSI*

(From the dog-tick, *Rhipicephalus sanguineus*, in preparation of Captain S. R. Christophers, I. M. S.)

FIGS. 1, 2, AND 3.—Forms showing prominent undulating membrane and blepharoplast anterior or lateral to the nucleus. Note the sharp posterior end and the presence of globules.

FIG. 4.—The same, with less prominent undulating membrane.

FIG. 5.—Form showing rounding-up of posterior end, also large globules.

FIG. 6.—Unequal longitudinal division.

PLATE 7.

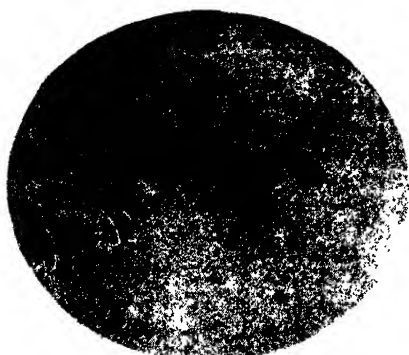


FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.



PLATE 8.



FIG. 1.



FIG. 2.

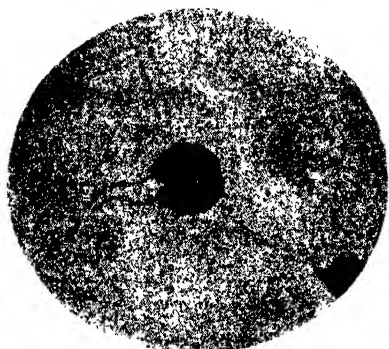


FIG. 3.

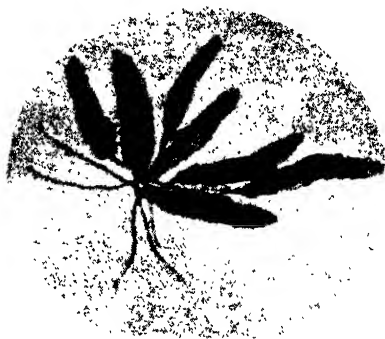


FIG. 4.

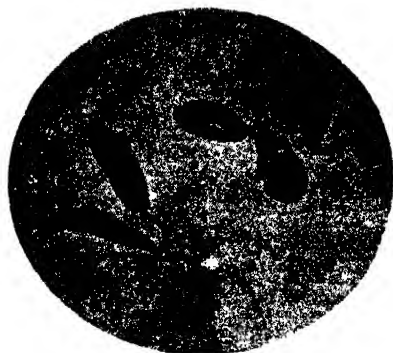


FIG. 5.



FIG. 6.



PLATE 9.



FIG. 1.



FIG. 2.



FIG. 5.

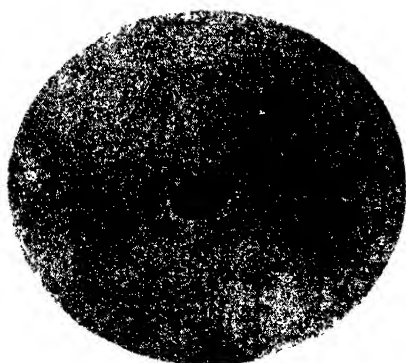


FIG. 3.



FIG. 4.





PLATE 10.



FIG. 1.



FIG. 2.



FIG. 3.

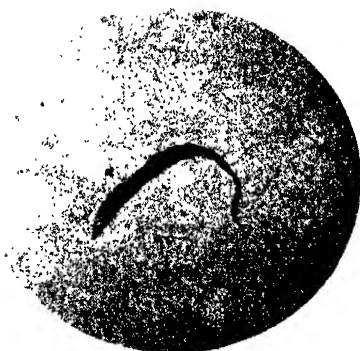


FIG. 4.



FIG. 5.



FIG. 6.



PLATE II.

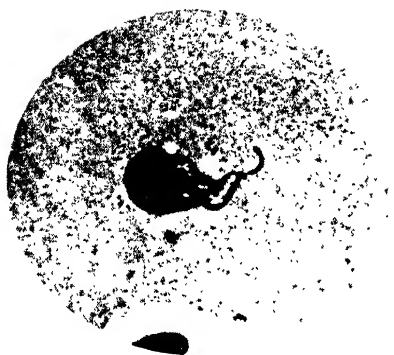


FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.



PLATE 12.



FIG. 1.



FIG. 2.

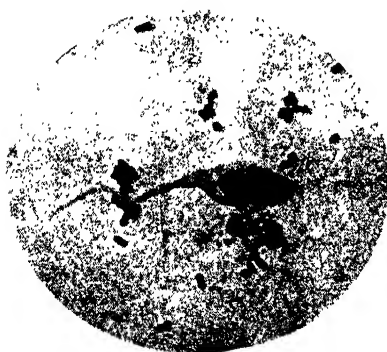


FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.



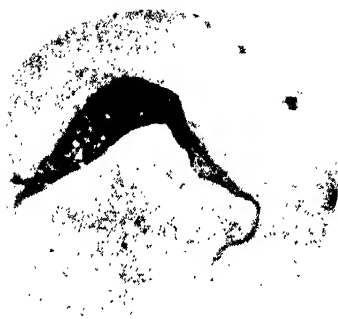


FIG. 1.

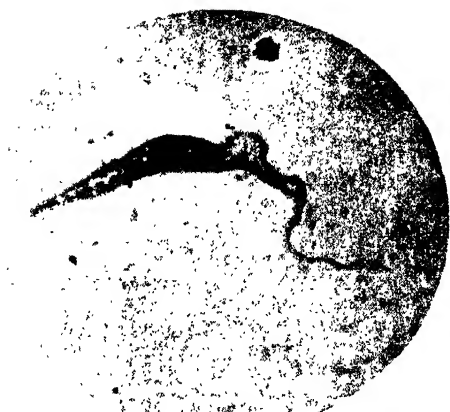


FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.





## Glucothionsäure in Leukocyten.

Von

J. A. Mandel und P. A. Levene.

(Aus dem chemischen Laboratorium der New York University and Bellevue Hospital Medical College und aus dem Rockefeller Institute for Medical Research, New York.)

(Eingegangen am 20. März 1907.)

Seit einiger Zeit ist es uns gelungen, die Glucothionsäure aus mehreren tierischen Organen darzustellen<sup>1)</sup>, und es dürfte deswegen diese Substanz als allgemeiner Gewebbestandteil anerkannt werden. Nun aber enthalten alle Organe eine gewisse Menge von Bindegewebe, und man kann nicht ohne weiteres ausschließen, daß die Glucothionsäure gerade aus diesem stamme, hauptsächlich weil es bekanntlich reich an mucoidähnlichen Substanzen ist.

Es war deswegen wünschenswert, solche Gewebe auf Vorhandensein der Glucothionsäure zu untersuchen, die keine Bindegewebsfasern enthalten. Als ein solches wurde Eiter gewählt, und dieser noch aus anderen Gründen; nämlich — erstlich war es längst bekannt, daß dauernde Eiterbildung amyloidartige Entartung verursacht, und es ist jüngst erwiesen worden, daß Amyloid ein Derivat einer Glucothionsäure ist<sup>2)</sup>; zweitens sind die Morphologen nicht ganz einig über die chemische Natur der Jodkörnchen in den Leukocyten. Manche Autoren sind der Ansicht, daß sie nicht aus Glykogen, sondern aus Mucoid bestehen.

Es wurde deswegen beschlossen, Eiter auf die Gegenwart der Glucothionsäure zu untersuchen. Um eine genügende Quantität desselben zu beschaffen, wurde an Pferden künstlich Pyothorax hervorgebracht. Dieses wurde auf folgendem Wege erreicht: 200 ccm einer 10proz. Suspension von Aleuronat in Stärkekleister

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<sup>1)</sup> Zeitschr. f. physiol. Chemie, **45**, 386, 1905.

<sup>2)</sup> Archiv f. experim. Pathol. u. Pharmakol. **25**, 195, 1898.

wurde in die Pleura unter aseptischen Bedingungen täglich drei Tage lang eingespritzt und am vierten Tage 50 ccm einer frischen Kultur von *Staphylococcus pyogenes aureus* mit Kuhvaccine auf demselben Wege eingeführt. Nach 8—10 Tagen wurden die Pferde getötet. In einem Versuch gelang es, auf diesem Wege, 6000 ccm einer breiartigen Masse von Leukocyten zu erhalten, und in einem anderen Versuche 2800 ccm.

Diese Masse wurde nach Zugabe von 600 g Kochsalz zwei Stunden lang gekocht und in üblicher Weise auf Glucothionsäure untersucht. Es gelang uns, eine Substanz zu erhalten, die alle Eigenschaften dieser Säure besaß und den folgenden Prozentgehalt an Stickstoff und Schwefel hatte:

0,2450 g Substanz erforderten bei einer Stickstoffbestimmung nach Kjeldahl 8,6 ccm Schwefelsäure (1 ccm dieser Schwefelsäure entsprach 0,00132 g N); also N = 4,66%.

0,2450 g Substanz gaben 0,0548 g BaSO<sub>4</sub>; also S = 3,07%.

## THE QUANTITATIVE ESTIMATION OF EXTRACTIVE AND PROTEIN PHOSPHORUS.<sup>1</sup>

By W. KOCH.

(From the Pathological Laboratory of the London County Asylums.)

(Received for publication, January 16, 1907.)

The different combinations of phosphorus to be found in a given tissue may be divided into three main groups:

1. *Protein phosphorus* or phosphorus in combination with protein, including nucleoprotein and phospho-proteins or nucleo-albumins, insoluble in water especially after treatment with alcohol;

2. *Lecithin and kephalin phosphorus* or phosphorus in combination with fat and a nitrogen complex, soluble in alcohol and ether, but insoluble in acid chloroform water;

3. *Extractive phosphorus*, including inorganic phosphates and the simpler combinations of phosphoric acid, such as glycerophosphoric acid, phytin or diethoxy-diphosphoric acid, and a number of related compounds as yet little investigated, all of which are soluble in water and partly soluble in dilute alcohol.

In a previous paper<sup>2</sup> a method for the estimation of lecithin and kephalin phosphorus was described. In the following pages are given methods for the determination of extractive and nuclein phosphorus, which can be carried on at the same time and with the same material as the lecithin estimation.

### EXTRACTIVE PHOSPHORUS.

A considerable portion of this form is found in the filtrate from the lipoids precipitated with acid chloroform as described in the above mentioned publication. Whether any of this phosphorus

<sup>1</sup> These methods were used in the investigation with H. S. Reed, published in vol. iii, p. 49, of this *Journal*.

<sup>2</sup> Koch and Woods: This *Journal*, i, p. 203, 1905.

is inorganic cannot be determined. Schulze<sup>1</sup> in several of his publications emphasized the fact that absolute alcohol and ether do not dissolve inorganic phosphates. He is dealing, however, with relatively dry plant tissues and not with moist animal tissues which necessarily dilute the alcohol. The separate estimation of inorganic phosphates has not been attempted in these methods, as the danger of hydrolyzing simple organic combinations of phosphoric acid seemed too great to promise reliable results.

The following table gives an idea of the amount of phosphorus, not lecithin or kephalin, dissolved out by alcohol and ether from brain tissues.

TABLE I.

PHOSPHORUS IN FILTRATE FROM LIPOID PRECIPITATE. ALCOHOL-ETHER-WATER-SOLUBLE PHOSPHORUS.

Number of Case.	In Per Cent of Dry Tissue.	In Per Cent of Total Extractive P.	In Per Cent of Total P.
34 ♀	0.68	37.8	4.8
35 ♀	0.63	32.1	4.5
44 ♀	0.75	36.0	5.4

The remaining portion of the extractive phosphorus is to be found in the portion of the tissues insoluble in alcohol and ether and must be removed by treatment with water to which a little chloroform has been added to prevent bacterial action. Noël Paton<sup>2</sup> recommends dilute acid for this extraction, but does not make it clear whether he altogether avoids the possibility of breaking up more complex substances. Control experiments have shown that in the case of the brain about five or six extractions are sufficient to remove all the phosphates that can be removed. The following table gives the results.

TABLE II.

WATER-SOLUBLE PHOSPHORUS IN RESIDUE INSOLUBLE IN ALCOHOL AND ETHER. ALCOHOL-ETHER-INSOLUBLE, WATER-SOLUBLE PHOSPHORUS.

Case.	In Per cent of Dry Tissue.	In Per Cent of Total Extractive P.	In Per Cent of Total P.
34 ♀	1.12	62.2	7.8
35 ♀	1.33	67.9	9.5
44 ♀	1.33	64.0	9.6

<sup>1</sup> Schulze, E.: *Zeitschr. f. physiol. Chem.*, xx, p. 225, 1904.

<sup>2</sup> Noël Paton, D.: *Report of Investigations on the Life History of the Salmon in Fresh Water*. Fishery Board for Scotland, p. 143, 1898.

## PROTEIN PHOSPHORUS.

The phosphorus compounds present in the tissues after extraction with alcohol, ether and water can only be nucleins, phospho-proteins and tricalcium phosphates. The latter compound is not usually supposed to be present in appreciable amount in tissues except under pathological conditions and can therefore be neglected in the case of brain tissues. If calcium is present it would be more likely to exist as a calcium protein compound. Extraction with dilute acid might be used where calcium phosphate is suspected but this procedure so swells the tissues that complete removal of the adhering liquid becomes very difficult. Besides there is the danger of rendering the alcohol-coagulated protein again soluble.

The following table gives some of the results:

TABLE III.

PHOSPHORUS IN INSOLUBLE RESIDUE. ALCOHOL-ETHER-WATER-INSOLUBLE  
OR PROTEIN PHOSPHORUS.

Case.	In Per cent of Dry Tissue.	In Per Cent of Total P.
34 ♀	0.81	5.6
35 ♀	0.86	6.1
44 ♀	0.85	6.1

A comparison of Tables I, II and III will show that about 80 per cent of the total phosphorus remains to be accounted for. This is represented by lipid phosphorus which, in the case of corpus callosum here analyzed, is present in large amount.

## DESCRIPTION OF METHOD.

About 10 grams of the moist tissue are extracted with alcohol and ether as directed in the paper on the "Estimation of the Lecithins." The *residue, insoluble in alcohol and ether*, is dried at 102° C. to constant weight, transferred to a 300 cc. Jena flask and extracted six times with about 100 cc. of water to each extraction. Every extraction should extend over 24 hours; plenty of chloroform must be added and the mixture occasionally shaken to prevent bacterial decomposition. The filtrates are evaporated in a platinum dish and dried to constant weight. The residue represents the *salts and extractives, insoluble in alcohol and ether*

*and soluble in water.* The dried residue is ignited in the platinum dish, surrounded by an outer larger platinum dish which is heated to bright redness, until a nearly white ash is obtained. If the inner dish does not come in direct contact with the outer dish there is no danger of volatilizing chlorids. This residue is the *alcohol-ether-insoluble, water-soluble ash*. The difference between this ash and the residue on evaporation gives the *alcohol-ether-insoluble, water-soluble, organic extractives*. The ash is moistened with 1.5 cc. of nitric acid, dissolved in water, diluted to 100 to 200 cc. and phosphorus estimation made by the molybdate method. This gives the *alcohol-ether-insoluble, water-soluble extractive phosphorus*. (Table II.)

The residue left above, insoluble in water after six extractions, is burned with nitric and sulphuric acids and the phosphorus estimated. In case calcium is present this must also be estimated in a separate sample. The phosphorus method is described in detail in a previous paper.<sup>1</sup> This phosphorus is called the *alcohol-ether-water-insoluble, or protein phosphorus*. (Table III.)

The alcohol and ether solutions obtained by the extraction of the moist tissue are treated as directed in the paper above referred to. If the emulsification and precipitation have been properly carried on, the solution in the 100 cc. graduated flask should be clear in two or three days. An excess of fat in the tissue interferes seriously with this clearing and had best be overcome by the presence of a large amount of chloroform (8 to 10 cc.) and the addition of 2 cc. instead of 1 cc. of hydrochloric acid. The amount of chloroform added must be *carefully measured and recorded*. After the solution has begun to clear and has been made up to the 100 cc. mark of the graduated flask, it is shaken and allowed to stand until the precipitate has settled. After settling the solution is filtered through a dry filter paper into a dry 100 cc. graduated cylinder. As much of the water as possible is decanted from the chloroform, but it is better not to pour any of the chloroform on the filter, as it may pass through and lipoids be thus lost. Instead of washing the chloroform containing the lipoids with acid water as previously directed, it is better to allow the filter to drain and then *read the volume of the filtrate*,

<sup>1</sup> Koch and Woods: *loc cit.*

which should be perfectly clear and transparent. An aliquot part of the filtrate, usually 80 cc., is evaporated in a platinum dish and dried to constant weight at 102° C. This gives the *extractives and salts soluble in alcohol, ether and water*. The residue is ignited as directed above and the ash is the *alcohol-ether-water-soluble ash* while the difference between this and the residue obtained at 102° C. represents the *alcohol-ether-water-soluble extractives*. The ash is again moistened with  $\frac{1}{2}$  cc. of nitric acid, diluted to 100 to 200 cc. and phosphorus estimated. This is the *alcohol-ether-water-soluble extractive phosphorus*. (Table I.)

## DISCUSSION AND CALCULATION.

In order to illustrate the method of calculation it is best to take a sample analysis as follows:

## RECORD OF ANALYTICAL RESULTS.

## Case 34 ♀ Corpus Callosum.

Water* . . . . . 70.37 per cent	Weight of sample . . . . .	9.9038 grams
1. Insoluble residue† . . . . . 0.68 " "	Weight of residue insoluble in alcohol and ether . . . . .	0.8886 "
	Weight of residue from six extractions . . . . .	0.0289 "
2. Lecithins . . . . . 4.00 " "	Weight of residue, insoluble in alcohol, ether and water . . . . .	0.8597 "
3. Cephalins . . . . . 4.27 " "	Phosphoric acid in this residue gave . . . . .	0.0085 " Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>
4a Extractives . . . . . 0.15 " "		
5a Extractives . . . . . 1.03 " "	Residue from six water extractions . . . . .	0.0289 grams
4b Ash . . . . . 0.14 " "	4b Ash on ignition . . . . .	0.0142 "
5b Ash . . . . . 0.45 " "		
6 Sulphur Compound* 1.40 " "	4a Extractives . . . . .	0.0147 "
	Phosphoric acid gave . . . . .	0.0117 " Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>
	Protein P . . . . . 0.024 per cent.	
	Total Extract P . . . . . 0.053 " "	

To precipitate lipoids 3 cc. chloroform were used. The filtrate measured 76 cc.; 70 cc. were evaporated.

Residue . . . . .	0.1060 grams
5b Ash on ignition . . . . .	0.0323 "
5a Extractives . . . . .	0.0737 "
Phosphoric acid in this residue gave . . . . .	0.0051 " Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>
2 Lecithin P gave . . . . .	0.0551 grams
3 Cephalin P gave . . . . .	0.0697 "

\* Separate estimation.

† This essentially represents the total proteids and any glycogen that may be present in the tissues.

The method of calculating the above results is as follows:

5a and 5b alcohol-ether-water-soluble extractives and ash are calculated to 97 cc. (thus correcting for the volume of the chloroform)

$$\begin{array}{rcl} 0.0737 \times 97 \times 100 & & 0.0323 \times 97 \times 100 \\ 70 \times 9.9038 & = 1.03; & 70 \times 9.9038 = 0.45 \end{array}$$

Alcohol-ether-water-soluble, extractive phosphorus.

$$\frac{0.0051 \times 97 \times 100 \times 62}{70 \times 9.9038 \times 222.7} = 0.020$$

The same method applies to the calculation of the alcohol-ether-water-soluble phosphorus. The filtrate obtained was only 74 cc., but as only 5 cc. of chloroform were used, the remaining watery solution must have remained clinging to the rather spongy mass of fat and chloroform. Some little solution goes to moisten the filter paper, but this partly counterbalances whatever chloroform may have gone into solution. This method of calculation is not absolutely accurate, but comes sufficiently near, considering the variations to which the material is liable in any case.

3. *The kephalin* receives a correction for the amount of phosphorus in the liquid clinging to the lipid precipitate, in this case  $95 - 74 = 21$  cc., equivalent to 0.06 milligram of phosphorus or 0.08 per cent of kephalin.

Theoretically the correction should be distributed between the lecithin and kephalin, the values of both of which it affects. As it is not improbable, however, that the water-soluble phosphoric acid derivatives with which we are here dealing form insoluble lead salts in ammoniacal alcohol solution, it was deemed best to apply the whole correction to the kephalin. The kephalin in the case of brain tissues receives a further correction for the phosphorus found in the sulphur compound.

In the light of later results it was found advisable to change the method for the estimation of kephalin outlined in a previous paper as follows:

*This Journal, i, p. 208, line 7. Substitute:* allow to remain on water-bath until there is no more smell of ammonia. The *flask* is then set aside to cool, after replacing the alcohol which has evaporated.

*p. 208, line 12. Read:* and the precipitate washed *once* with hot alcohol.

*p. 208, line 21. Experience* has shown that it is preferable to burn the filter paper with the precipitate.

In the muscle tissues of animals containing a solid fat the correction for the kephalin on account of filtrate adhering to lipid precipitate may become rather large. It can, however, be easily reduced by making the precipitate in a larger flask (200 cc. or 400 cc.) and thus diluting the adhering filtrate.

The method above outlined should be capable of more general application to normal and pathological material. Such a study on the nervous system is soon to be published. This investigation was aided by grant from the Rockefeller Institute for Medical Research.



## GASTRIC PERISTALSIS IN RABBITS UNDER NORMAL AND SOME EXPERIMENTAL CONDITIONS.

By JOHN AUER.

[From the Rockefeller Institute for Medical Research and the Physiological Laboratory, Harvard Medical School.]

### INTRODUCTION.

UNTIL quite recently knowledge of the behavior of normal gastric peristalsis was derived from studies by various methods, all of which were carried out under more or less distinctly pathological conditions. These methods were as follows: (1) Observation of the excised stomach placed in a moist, warm chamber (Hofmeister and Schütz<sup>1</sup>). The abnormality of this procedure is evident and requires no comment. (2) Observation of the stomach in the living animal with opened abdomen without anæsthesia (Wepfer,<sup>2</sup> Schwartz<sup>3</sup>) or under the influence of morphine or curare (Rossbach<sup>4</sup>). We know now that pain and rage as well as morphine and curare modify the behavior of normal peristalsis. The profound effect which opening of the abdomen exerts upon peristalsis will be discussed later. (3) Study of the peristalsis through a gastric fistula in man and animals by introducing into the stomach a thermometer (Beaumont<sup>5</sup>), a manometer (Uffelman<sup>6</sup>) or a balloon connected with a graphic apparatus (Ducceschi<sup>7</sup>). The adhesions of the stomach to the abnormal wall and the presence of foreign bodies in the stomach surely influence normal peristalsis. (4) Peroral introduction of a stomach

<sup>1</sup> HOFMEISTER and SCHÜTZ: *Archiv für experimentelle Pathologie und Pharmacologie*, 1886, xx, p. 7.

<sup>2</sup> WEPFER: *Historia cicutæ aquaticæ*, Basel, 1679, p. 152.

<sup>3</sup> SCHWARTZ: *HALLER'S Dissertationes anatomicæ*, Göttingen, 1746, i, p. 337.

<sup>4</sup> ROSSBACH: *Deutsches Archiv für klinische Medicin*, 1890, xlv, p. 296.

<sup>5</sup> BEAUMONT: *Physiology of digestion*, Burlington, 1847.

<sup>6</sup> UFFELMAN: *Deutsches Archiv für klinische Medicin*, 1877, xx, p. 546.

<sup>7</sup> DUCCESCHI: *LUCIANI'S Physiologie des Menschen*. Translated by BAGLIONI and WINTERSEIN, 1906, ii, pp. 163 *et seq.*

tube, the tip of which is provided with a balloon (Morat<sup>1</sup>). Besides the fact that we have here again a foreign body in the stomach, the presence of the tube in pharynx and œsophagus and the reflexes it produces are probably not without modifying influences upon the normal course of gastric peristalsis. Moreover, tracings so obtained without any other control are open to a variety of interpretations.

The only method which can claim to investigate normal peristalsis under normal conditions is the fluoroscopic inspection of the stomach after giving the animal food mixed with bismuth. As is well known, this method has been extensively employed by W. B. Cannon<sup>2</sup> for the last ten years, and the numerous results which it brought to light need not be pointed out to readers of this journal. In these studies the observations were mostly made on the stomachs of cats. A disadvantage of this method is the fact that it demands complex apparatus, and furthermore the method requires also an investigator who is an expert in this mode of observation.

Considering the numerous researches which deal with gastric peristalsis, it is somewhat surprising that a method was overlooked by means of which stomach motility could be studied in a common laboratory animal without any operation, and under conditions that are as physiological as any method can give that demands fixation of the animal. In the rabbit mere inspection reveals gastric peristalsis. In the literature no statement was found indicating this fact. On the contrary, many writers point out especially the tardiness or lack of peristalsis of the rabbit's stomach. But these writers studied the stomach in the opened abdomen, where its motility is indeed strikingly retarded, if not entirely absent.

#### NORMAL PERISTALSIS OF THE RABBIT'S STOMACH.

**Methods of observation.** — The animals were observed under normal conditions; the only abnormal state was their extension and fixation on a holder. They were placed on cotton and well covered to prevent loss of heat as much as possible. Disturbing and exciting influences were avoided. No narcotics were administered except in experiments devoted to the study of their effects.

The observations were carried out, in the first place, by simple inspection. With a little practice any one may recognize the stomach

<sup>1</sup> MORAT: Archives de physiologie, 1893, v, p. 142.

<sup>2</sup> CANNON, W. B.: Science, June 11, 1897, p. 902; This journal, 1898, i, p. 359.

waves and follow out their course. It is a great advantage that a number of persons may observe the peristaltic movements at the same time, and individual subjective observations can thus be satisfactorily controlled. Besides simple inspection, tracings of the gastric movements were obtained. These tracings have the advantage that their interpretation was controlled by the inspection method.

*Inspection.*—If a rabbit, well fed shortly before the experiment, is stretched on its back and the hair of the upper abdominal region cut short, a large part of the stomach can easily be outlined by inspection and by palpation. The position occupied is usually either diagonally from the right chondro-xiphoid region downward and to the left, or the organ lies transversely. Full-grown rabbits are preferable, for their stomachs are larger and a greater area is available for observation, due to a perhaps normal gastropotosis.

Inspection of the stomach area for the first few minutes reveals no motion (Fig. 3, *a*), but after three to ten minutes a shallow depression may be seen apparently arising at the junction of the fundic and middle thirds of the stomach. This depression courses slowly over the viscus from left to right, increasing moderately in depth as the pyloric third is approached. The initial depression on the abdominal wall of the rabbit over the stomach apparently does not reach the greater curvature. Whether this wave involves the lesser curvature cannot be stated, for this portion of the stomach is hidden from sight by a lobe of the liver. Preceding the depression is a bulging, which increases and reaches its maximum at the beginning of the pyloric third. On reaching this point the depression often seems to pause for an appreciable period of time, and then a contraction of the bulging pyloric third sets in. This latter contraction does not seem peristaltic, but apparently occurs more or less as a whole; the bulging sinks away without showing any peristaltic wave. During this sinking the contraction causing the bulging relaxes, and the contents of the pyloric third may often be seen forced partially into the middle third of the stomach again, thus simulating a short anti-peristaltic wave. At other times, especially in young rabbits, and when the stomach was moderately dislocated downward in order to render the pyloric third more completely visible, the contraction of this portion of the stomach seemed to be caused by a peristaltic wave which traversed the bulging.

When peristalsis is fully established, the prominence of the pyloric third at the end of a gastric wave is very marked, and its peculiar

contraction may then be best noted. The contraction may reduce the mass to the same level occupied by that stomach region before diastole (Fig. 1, *a*), or the systole may be so powerful that the pyloric third disappears entirely from view (Fig. 1, *b*). During a contraction of the latter type a bubbling sound is often audible.

The waves increase slowly in vigor and frequency, and are most marked about one to two hours after feeding (Fig. 1, *a*, *b*). Then the constriction at the beginning of the pyloric third and the bulging of that portion become extremely marked. In many instances the constriction was so marked that separation of this part from the rest of the stomach cavity seemed to be produced, the organ showing an hourglass shape. This constriction was a few millimetres in width at its base, and either maintained its position and strength during the ensuing contraction of the pyloric third or relaxed during it.

*Pyloric sounds.* — If the powerful constriction described above was more or less maintained, a musical gurgling sound was frequently heard during the contraction of the pyloric third. This sound seemed to be produced in the right hypochondriac region, and on lightly pressing a finger beneath the pyloric portion of the stomach over the duodenum, a fine bubbling thrill of varying length and intensity could be easily felt. This thrill occurred at irregular intervals, and seemed undoubtedly to be produced by the expulsion of liquid material from the stomach into the gas-containing duodenum. Palpation frequently showed a thrill in the duodenal region, when the unaided ear heard no sound, and when the constriction above noted was not extreme. It must be mentioned that a loop of the cæcum passes just beneath the pyloric third of the stomach, and in this loop cæcal sounds may be heard and felt. Their dull, popping character and coarse thrill is quite different from the rather musical, high-pitched sound and fine thrill produced by the expulsion of material through the pyloric sphincter.

*Graphic registration.* — The frequency of the waves varied from one to five or more per minute; two waves crossing the stomach at the same time could always be seen when peristalsis was active. In order to count them for long periods of time graphic registration was used.

This was easily accomplished by placing a Marey receiving tambour, about four centimetres in diameter, over the pyloric third of the stomach, and then connecting with a Marey writing tambour which traced the volume curve of the stomach area covered on smoked paper. The receiving

tambour was held in place by four pieces of tape radiating from the tambour stem, the tape ends passing through holes in the rabbit board where they were fixed by plugs. The position of the tambour itself was marked on the abdominal wall with pencil, so as to permit readjustment when dislodged by movements of the animal. In this connection it may be permitted to state that considerable patience is often required with some rabbits, for active movements usually require rearrangement of the receiving tambour. After preparing the animal in this fashion it was covered with cloths and cotton wool, so as to prevent loss of heat as much as possible.

In the tracings the *upward* direction of the curve means an increase in volume of the stomach area covered by the tambour, a diastole; the *downward* movement means a diminution in volume of that area, a systole.

By this method not only the stomach waves are written, but the respirations are also shown as oscillations superimposed on the stomach waves. With delicate adjustment of the writing lever the

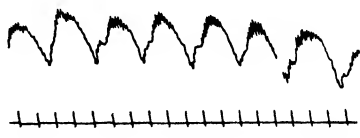


FIGURE 1 a. — All the tracings are taken from the pyloric third of the rabbit's stomach by means of tambours. The large waves are stomach waves, the rising limb representing an increase in volume, diastole; the descending limb representing a diminution of volume, systole, of the pyloric third. The smaller curves superimposed on the larger are respiratory oscillations. Time marks represent six seconds. Tracing taken twenty minutes after feeding.

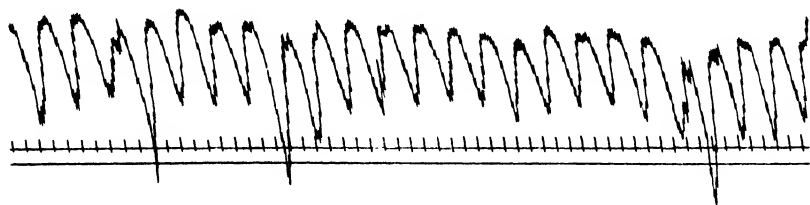


FIGURE 1 b. — Same animal eighty minutes after feeding. The three strong contractions are to be noted; pyloric sounds as a rule are associated with them.

heart beats are shown as small notches on the respiratory oscillations. The pyloric third was usually chosen for registration because the waves are there most marked. The tracings bring out well the points already described, and emphasize the almost machine-like regularity of the waves, which occur one or two hours after feeding. Then there is usually only a slight pause, or none at all, between the rhythmic play of systole and diastole (Figs. 1, a, 2, b, 3, c). If a considerable

length of time has elapsed since the last feeding and when peristalsis is slow, the pause between systole and diastole may be marked (Figs. 1, *c*, 3, *a*) and the waves do not occur with such marked regularity, both as to time and strength of contraction.

The length of time necessary for a wave to traverse the stomach

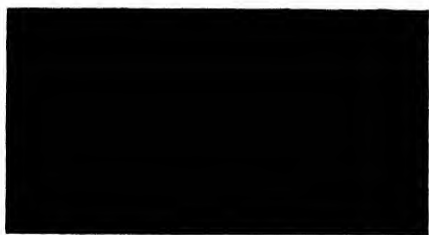


FIGURE 1 *c*. — Five hours after feeding. Taken from another rabbit which showed rapid respiration.

starting time cannot be noted, because the depression caused by a beginning gastric wave is probably so slight that the abdominal wall shows no sign of it. Bearing this in mind, it may be stated that the waves take approximately about twenty seconds to cross the stomach. The contraction of the pyloric third, from the beginning of diastole

to the end of systole, consumes about fifteen seconds. This time interval is fairly constant, as an examination of the tracings will show.

As already stated, the site of origin of a gastric wave cannot be determined with accuracy. Inspection shows the first sign of a depression on the anterior surface of the stomach, about at the junction of the fundic and middle thirds. This corresponds about to the region below the entrance of the œsophagus. When the receiving tambour is placed over the fundus, changes in volume are registered; but this can by no means be interpreted as due to a wave starting at that place; it might well represent only the bulging caused by the transmission of pressure produced by a wave beginning nearer the pylorus. The method described can give no definite answer to the question, whether or not gastric waves begin in the fundus.

When the abdomen of a rabbit is opened so as to identify the different portions of the stomach seen through the intact abdominal wall, it is found that the pyloric third is composed of two well-defined divisions, an antrum and a preantrum. The marked bulging which the abdominal wall shows towards the end of a gastric wave, as described in the preceding pages, is caused by the filling of the preantral portion. This preantral division is well marked off from the rest of the stomach by a pouching which occurs both on the lesser and greater curvatures, the one on the greater curvature being the longer and larger of the two; the preantrum thus forms a knee

connecting the stomach with the antrum. But these outpocketings are not the only features that serve to differentiate this region; its musculature is thick, practically as thick as that of the antrum itself, and the border of the preantral bag is quite sharply marked off from the thin musculature of the fundic part of the stomach. The musculature of the preantrum shows strong circular fibres, some of which apparently insert in tendinous patches, of which each surface, anterior and posterior, shows one. The preantrum is flattened anteroposteriorly, and the level of the two surfaces is about that of the stomach proper.

The antrum itself is very muscular, and forms a cone-shaped cap, closing the preantral cavity. Between antrum and preantrum is a definite constriction marking the *antral* sphincter; between preantrum and the rest of the stomach is another perfectly definite constriction, and at this region the preantral musculature abruptly loses its thickness, its border forming the *preantral* sphincter.

Inspection of a rabbit's abdomen, as a rule, shows only the preantral portion of the pyloric third of the stomach; the antrum is too deeply located to affect during its activity the surface conformation of the abdomen under ordinary conditions.

**Discussion.** — From the description given above, it will be seen that the preantral region of the rabbit's stomach is definitely differentiated anatomically, and possibly has also a different function. The literature does not contain definite statements on the preantrum. Hofmeister and Schütz,<sup>1</sup> it is true, speak of a "preantral constriction," which marks the end of the first phase of their "Peristole," but it is evident that they only mean to localize the site of the constriction by this term; there is no evidence that they meant to differentiate a preantrum from the antrum. Cannon,<sup>2</sup> in a diagram of the cat's stomach, distinguishes a preantrum from the antrum, but no special statement is made regarding its structure and function. Ellenberger and Baum<sup>3</sup> make no mention of a preantrum in the dog. The textbooks on human anatomy describe no preantral region. For the rabbit itself this anatomical differentiation of the pyloric third of the stomach into an antrum and preantrum seems to be unknown; Krause<sup>4</sup> states nothing bearing on this question. Nor do

<sup>1</sup> HOFMEISTER and SCHÜTZ: *Loc. cit.*

<sup>2</sup> CANNON: This journal, 1898, i, p. 364.

<sup>3</sup> ELLENBERGER and BAUM: *Anatomie des Hundes*, 1891, p. 290.

<sup>4</sup> KRAUSE: *Die Anatomie des Kaninchens*, 1884.

Oppel<sup>1</sup> and Wiedersheim<sup>2</sup> mention or give a drawing of the preantrum in rabbits.

It is interesting to note, however, that the behavior of the rabbit's *preantrum* during a gastric wave tallies quite well with the description Hofmeister and Schütz<sup>3</sup> give of the rôle played by the *antrum* in the dog. Those authors state that a gastric wave, as seen in the excised stomach of a dog, consists of two phases. During the first phase a peristaltic wave, increasing in intensity as it progresses, ends in a deep preantral constriction. During the second phase the sphincter antri contracts powerfully, the preantral constriction relaxing meanwhile, so that the stomach assumes an hourglass shape, the antral cavity being separated from the rest of the stomach; then the antrum contracts, not peristaltically, but more or less as a whole. It is unfortunate that the data at hand do not justify any definite statement regarding the behavior of the rabbit's antrum during a gastric cycle.

Moreover it must be mentioned that Hofmeister and Schütz's description of gastric peristalsis, while corroborating Beaumont's<sup>4</sup> account, has not in its turn been corroborated by more recent investigators. Rossbach,<sup>5</sup> Cannon,<sup>6</sup> Roux and Balthazard,<sup>7</sup> all state that the gastric wave sweeps peristaltically over the antrum in cats, dogs, and man.

Whether in the rabbit the antrum behaves like its preantrum, and whether there is a difference in the peristaltic contraction of the stomach between herbivorous and carnivorous animals, are problems whose solution must be left to future studies.

#### GASTRIC PERISTALSIS UNDER SOME EXPERIMENTAL CONDITIONS.

This method lends itself readily to the study of gastric motility under various experimental conditions, and some of the facts obtained will now be described.

<sup>1</sup> OPPEL: Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbelthiere, 1896, i, p. 386.

<sup>2</sup> WIEDERSHEIM: Vergleichende Anatomie der Wirbelthiere, 1902, p. 376.

<sup>3</sup> HOFMEISTER and SCHÜTZ: Archiv für experimentelle Pathologie und Pharmakologie, 1886, xx, p. 8.

<sup>4</sup> BEAUMONT: *Loc. cit.*

<sup>5</sup> ROSSBACH: Deutsches Archiv für klinische Medicin, 1890, xlv, p. 296.

<sup>6</sup> CANNON: This journal, 1898, i, p. 367.

<sup>7</sup> ROUX et BALTHAZARD: Comptes rendus de la société de biologie, 1897, iv, p. 705; Archives de physiologie, 1898, xxx, p. 88.



**Fasting.** — If a well-fed animal, whose stomach motility has been noted, is starved from twelve to twenty-four hours, the peristaltic waves are greatly reduced in strength and may be absent entirely. Full-grown animals must be used for this study, because a twenty-four-hour fast reduces the volume of the stomach, so that in young rabbits the pyloric third is largely hidden by the right costal arch. In older rabbits the viscus is larger, lies lower in the abdominal cavity, and fasting for a moderate time does not markedly decrease the stomach surface available for study. This reduction, or cessation of stomach movements during fasting, has been noted by many observers in different animals. The cessation is particularly interesting in rabbits, because this animal's stomach is never empty; after a fast of ten days the stomach is considerably reduced in size, but it still contains a fair amount of material.



FIGURE 2a. — Stomach was moderately distended with air before tracing was taken. No movements visible before distention, as animal was starved.



FIGURE 2b. — Air was removed from the stomach per tube: diminution of gastric waves.

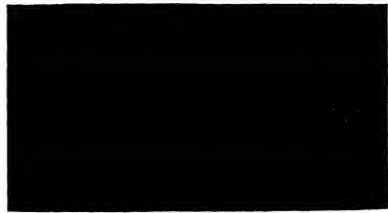


FIGURE 2c. — Same animal. Stomach re-distended with air: increase in strength of waves.

**Distention.** — From the above it would seem as if a certain distention were a factor in the production of the peristalsis. This supposition was established by an experimental test. When the stomachs of starving rabbits, which show only slight or no gastric motility, were distended with air or water, peristaltic waves arose, or were increased, if they had been present before. Figure 2, a, shows strong and regular gastric pulsations obtained from the pyloric third, after moderate distention of the viscus with air introduced per stomach tube; before the distention no waves were visible. Some time after distention, the air was removed as much as possible through a catheter passed into the stomach; the stomach waves were again greatly

reduced (Fig. 2, *b*). On redistention with air (catheter), vigorous peristalsis was re-established (Fig. 2, *c*). The distention must be moderate; the amount varies with each animal. Too much pressure not only fails to cause increased activity, but reduces whatever motility existed before distention; and if too little air or water is introduced the increase may be only slight or absent entirely. After distention with air pyloric sounds frequently occur, and by this method they may be produced at will.

This distention reflex has been described by Schütz,<sup>1</sup> who used the dog's excised stomach, and by Ducceschi.<sup>2</sup> The latter gives tracings illustrating the primary increase on distention and the decrease in strength of the contractions by overdistention of the dog's stomach.

Distention, however, is probably not the only factor in producing strong peristalsis. It was stated above that peristalsis of the rabbit's stomach was most vigorous one to two hours after feeding, and that the increase in vigor was gradual (Fig. 1 *a*, *b*). If the distention were the sole cause of the peristalsis, it should set in immediately after feeding, since it is then that the chief distention takes place; unless it is assumed that the stomach volume is considerably augmented by the formation of gases in some fashion or other within the stomach during digestion. However, the view expressed by some writers, that the products of digestion assist in stimulating the stomach movements possibly explains the gradual increase in a more plausible fashion.

#### CONDITIONS INHIBITING PERISTALSIS.

The stomach movements are easily inhibited by a variety of influences.

**Psychic influence.** — The necessary handling to which the rabbit is subjected when being tied on the board stops gastric peristalsis for a variable length of time, as has already been mentioned (Fig. 3, *a*). Within ten minutes, as a rule, movements again appear. But if the animal be startled in any way, or if it struggles, motion is again abolished for some time. Occasionally the stomach stops moving for no cause appreciable to the observer. The influence of emotions upon gastric peristalsis is not new; Cannon<sup>3</sup> has found that rage,

<sup>1</sup> SCHÜTZ: *Archiv für experimentelle Pathologie und Pharmakologie*, 1886, xxi, p. 343.

<sup>2</sup> See LUCIANI's *Fisiologie del' Uomo*, 1901, i, p. 680, for tracings.

<sup>3</sup> CANNON: *Loc. cit.*, p. 380.

fright, anxiety, abolish movements of the stomach in the cat; and Rossbach<sup>1</sup> has noted stoppage of intestinal peristalsis after the same causes in the human subject.



FIGURE 3 *a* — Tracing taken shortly after animal was stretched out on board. Note the initial inhibition (only partly shown in tracing).

**Ether.** — When ether is given continuously to a rabbit, advancing the cone very slowly toward the animal and avoiding respiratory stoppage, the first effect, as a rule, is cessation of peristalsis. This inhibition of gastric peristalsis lasts from thirty seconds to several

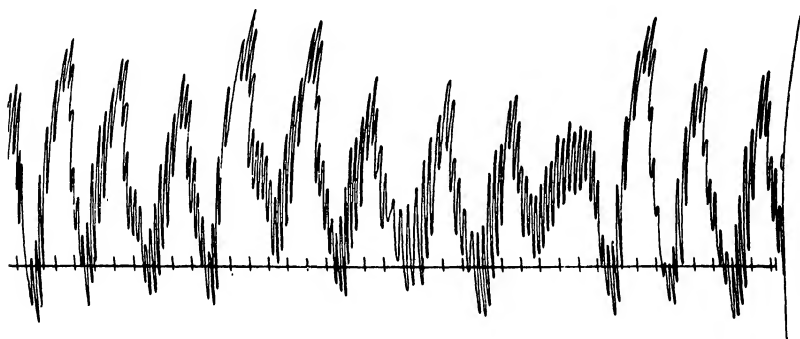


FIGURE 3 *b*. — Same animal now fully anesthetized by ether. The peristalsis is now more regular and powerful than before anesthesia.

minutes, respiration continuing uninterruptedly with careful administration of the ether (Fig. 3, *a*). This naso-gastric inhibitory reflex is thus seen to be more sensitive than the well-known naso-respiratory inhibitory reflex described by Kratschmer<sup>2</sup> and by Holmgren. Not all animals, however, show this period of inhibition at the beginning of ether anesthesia. (Some of these refractory animals suffered from a moderate nasal discharge, and it is possible that in those cases in-

<sup>1</sup> ROSSBACH: *Deutsches Archiv für klinische Medizin*, 1890, xlv, p. 326.

<sup>2</sup> KRATSCHMER: *Sitzungsberichte der Wiener Akademie, math.-phys. Kl. 2 Abt.*, 1870, lxii, p. 147.

flammation of the nasal mucous membranes was the cause of failure.) When ether was administered through a tracheal cannula, no inhibition was caused.

After the more or less long period of inhibition, gastric peristalsis is re-established; at first it is somewhat irregular, but later it is at



FIGURE 3 c. — Shows inhibition of gastric peristalsis when ether was started. Note slight effect on respiration.

least as regular as before the anæsthesia. This regularity is maintained even when the corneal reflex is absent (Fig. 3, *b*). During complete ether anæsthesia, therefore, gastric peristalsis is not abolished. In numerous instances, while the animal was completely under ether, the peristalsis was even more regular than before the anæsthetic was given (Fig. 3, *b*).

The course just described above is the rule; but there are exceptions. Ether anæsthesia sometimes retards the peristalsis and renders it irregular; periods of gastric quiet are occasionally interrupted by a series of waves of practically normal strength and duration. This course, however, forms an exception, and its character closely approaches that usually seen during chloroform anæsthesia.

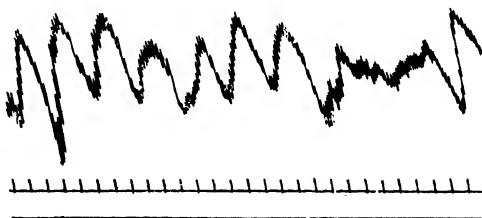


FIGURE 4 a. — Tracing taken under normal conditions. Note short inhibition of gastric waves.

**Chloroform.** — When a rabbit is allowed to inhale chloroform, at first in a very dilute form so as to avoid struggles of the animal, the initial effect is inhibition of gastric movements, respiration not being necessarily affected. The inhibition lasts a variable length of time, in general much longer than with ether. Then a few apparently normal waves appear, to be succeeded again by a period of inhibition. This play is repeated, inhibition alternating with the appearance of a few gastric waves (Figs. 4, *b*, *c*). These gastric waves are not weak, but are practically normal in strength. This course may be considered the type for chloroform. Here, again, there are exceptions to the rule. After induction of anæsthesia some rabbits may

fail to show a single gastric wave perceptible to the tambour; in others chloroform produces irregular waves throughout the anæsthesia. In general, however, the difference between ether and chloroform in their effect upon gastric peristalsis is well marked.



FIGURE 4 b.—Same animal. Chloroform has been administered twenty minutes.

The administration of chloroform requires more care than that of ether, and the stage of full anæsthesia is more difficult to judge.

With chloroform the lid reflex in rabbits is only abolished shortly before onset of rapid extensive respirations, associated with a high-pitched crying of the animal, which marks the beginning of respiratory failure.<sup>1</sup>

**The inhibitory effects of laparotomy.**—It has been shown that gastric peristalsis, as a rule, is active when a rabbit is anæsthetized with ether. If the abdomen of such an animal is now opened in the middle line and the stomach inspected, not a sign of motion will be detected. The organ which a few moments before showed active powerful waves lies there inert,



FIGURE 4 c.—Same animal. Under chloroform fifty minutes.

no motion can be seen. Well might Johannes Müller<sup>2</sup> say, "Die peristaltischen Bewegungen des Magens habe ich nie deutlich gesehen. . . ." If the viscus is allowed to become dry, slight waves may occasionally appear. Opening the peritoneal cavity under 0.9 per cent saline solution kept at a temperature of about 39° does not alter the picture; the stomach shows no movements worth mentioning. Stomach motility is lost as long as the peritoneal cavity remains open. If the abdominal wound be closed and the stomach area inspected after a few hours, gastric peristalsis may be observed

<sup>1</sup> It is worth noting that under ether as well as under chloroform the lid and corneal reflexes do not disappear at the same time; the corneal reflex seems the more sensitive and fails, while the lid reflex may still be active.

<sup>2</sup> MÜLLER: *Handbuch der Physiologie des Menschen*, dritte Auflage, 1838, i, p. 499.

again. *Mere opening of the abdomen, therefore, exerts a profound inhibitory effect of long duration upon the stomach.*

It is obvious from the foregoing that any method which demands opening of the peritoneal cavity for the study of stomach motility is incapable, at least in the rabbit, of throwing light upon the normal physiological behavior of the stomach.

Most investigators do not seem aware of the profound inhibition which exposure of the abdominal viscera produces. As far as I know Pal<sup>1</sup> is the only author who has called attention to the inhibition which abdominal section produces. But while he clearly and sharply notes the strong inhibitory effect of this operation upon the small and large intestines, only scanty attention is paid to the stomach.

It must be pointed out that Pal's method of experimentation did not entirely justify his conclusions, at least as far as the stomach is concerned. He experimented upon curarized animals, and observed the motility of the gastro-intestinal canal during cessation of artificial respiration. The cessation of artificial respiration is not an indifferent factor, as can be seen from the following observations made in the present investigation. In a rabbit fully curarized by intravenous injections of curarine, and kept alive by artificial respiration, the stomach shows excellent and regular peristalsis. But if artificial respiration be stopped, stomach peristalsis ceases at once, or within a few seconds. If artificial respiration be resumed in one to two minutes, gastric peristalsis will not set in until about two to four minutes have elapsed. This shows that interruption of artificial respiration in curarized rabbits stops gastric peristalsis. The stoppage of the peristalsis in Pal's experiments, at least as far as the stomach is concerned, could therefore have been due as much to the cessation of the artificial respiration as to the opening of the abdomen.

#### SUMMARY.

1. Gastric peristalsis may be observed in a well-fed rabbit without any operative interference whatsoever, by mere inspection of the epigastrium.

2. These movements are easily registered by placing a tambour

<sup>1</sup> PAL, J.: STRICKER'S Arbeiten aus dem Institut für experimentelle Pathologie der Wiener Universität, 1890, p. 31.

over the stomach area to be studied and connecting it with a writing tambour.

3. There is a striking difference between the effects of ether and chloroform upon gastric peristalsis; ether does not, as a rule, abolish gastric peristalsis, chloroform considerably reduces the stomach motility.

4. Opening the peritoneal cavity inhibits stomach peristalsis completely. This remarkable inhibition remains in force as long as the abdominal cavity is open; closure of the cavity is followed in a few hours by a return of gastric peristalsis.

5. Fasting reduces or completely abolishes stomach peristalsis.

6. Distention of the fasting stomach with air or water causes active peristalsis.

7. Curarization does not affect gastric peristalsis as long as artificial respiration is maintained. Stoppage of lung ventilation causes almost immediate cessation of stomach movements.

8. Temporary inhibition of gastric motility is produced easily by a variety of causes: handling of the animal, fright, struggles, irritating odors. Ether and chloroform vapors inhibit the stomach movements without causing respiratory stoppage.

9. Pyloric sounds in the rabbit are apparently produced by the expulsion of liquid material into the gas-containing duodenum; these sounds occur at irregular intervals, and when peristalsis is well established.

10. The rabbit's stomach shows a well-defined preantrum and preantral sphincter.

It is with great pleasure that I acknowledge the invaluable help and stimulating counsel of Dr. S. J. Meltzer during this investigation.





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**Observations on a rabbit for thirty months after the removal  
of the superior cervical ganglion.**

**By S. J. MELTZER.**

[*From the Rockefeller Institute for Medical Research.*]

Langendorff<sup>1</sup> reported that in one experiment on a cat one hundred and five days after the removal of the superior cervical ganglion, the paralytic symptoms of the eye disappeared, and stimulation of the cervical sympathetic nerve caused the typical effects. Microscopically no nerve cells could be detected, and Langendorff assumed that there was a union between the pre-ganglionic and postganglionic nerve fibers. Langley,<sup>2</sup> on the other hand, reported, about one year before Langendorff, an experiment on a cat in which twenty-three months after the removal of the superior cervical ganglion stimulation of the cervical sympathetic did not produce the usual effects, and on microscopical examination some postganglionic nerve fibers were found to have been regenerated; but there were neither nerve cells nor any union between the post-ganglionic and preganglionic nerve fibers. Later Langley and Anderson<sup>3</sup> repeated the experiment on eight cats. In six of the animals, which lived between one hundred and eighty-three and four hundred and seventy-six days, the paralytic symptoms remained permanent, and stimulation of the cervical sympathetic caused no effect. In two of the cases there was some decrease in the paralytic symptoms, and stimulation of the cervical sympathetic caused some effect, but microscopical examination showed that in both cases not all of the nerve cells had been removed.

All the above experiments were made on cats, which have a large ganglion. The gap between the postganglionic and pre-ganglionic nerve fibers in the cat is nearly one centimeter. In the

<sup>1</sup> Langendorff: *Centralblatt für Physiologie*, xv, 483, 1901. The number of days is quoted here from Langley and Anderson; it is not mentioned in the *Centralblatt*.

<sup>2</sup> Langley: *Journal of Physiology*, xxv, 417, 1900.

<sup>3</sup> Langley and Anderson: *Journal of Physiology*, xxxi, 383, 1904.

rabbit the ganglion is barely three millimeters long, and there might perhaps be a better chance for a final union of the nerve fibers of the two poles of the ganglion. I am going to report here briefly some observations made on a rabbit which lived over thirty months after the removal of the superior cervical ganglion.

Full grown, grey, male rabbit. Left superior cervical ganglion removed October 14, 1904. Animal died April 23, 1907.

Soon after the removal of the ganglion the left pupil became quite small; a few days later it became somewhat wider again, and some weeks later it became constricted to about the original size after the operation and retained this size permanently until death. The blood vessels of the left ear, which became wider after the removal of the ganglion, gradually assumed the size of the vessels of the other ear and remained in that state permanently. *During the last eighteen months the blood vessels of both ears were never very wide and showed but little of the usual rhythmical changes.*

We<sup>1</sup> have shown that after removal of the ganglion, a subcutaneous injection or an instillation of adrenalin into the conjunctival sacs of the rabbit causes a dilatation of the pupil on the side from which the ganglion was removed. This *biological test for the absence of the ganglion* was frequently made within the two and a half years of the animal's life and it was found that a *subcutaneous or intramuscular injection or an instillation of adrenalin invariably caused a long lasting dilatation of the left pupil*. This test seemed to prove satisfactorily that the ganglion was not regenerated, or at least the postganglionic and preganglionic nerve fibres did not grow together. To obtain further proof, twenty-eight months after the removal of the ganglion the cervical sympathetic nerves of both sides were exposed and stimulated with induction currents. While stimulation of the right sympathetic easily caused the usual effects upon the ear vessels and pupil of the corresponding side, *stimulation of the left cervical sympathetic caused no changes whatsoever in the left pupil or in the vessels of the left ear.*<sup>2</sup>

<sup>1</sup> S. J. Meltzer and Clara Meltzer Auer: *American Journal of Physiology*, xi, 28, 1904.

<sup>2</sup> This experiment was carried out in the presence of Drs. Flexner, Opie and Carrel.

During the last twelve months there were, however, a few changes which deserve to be mentioned. In the first place the dilatation of the left pupil never attained the same degree as during the first period. Further an intramuscular injection of adrenalin, which in the early period brought out the dilatation of the pupil within two or three minutes,<sup>1</sup> now developed its effect very slowly. Finally the constricting effect of eserine was only partly overcome by an injection or instillation of adrenalin, whereas in the early period the effect of eserine was completely overcome by adrenalin. Apparently the relations of adrenalin to the dilator pupillæ had somehow undergone some changes. Local stimulation of the iris was not tested.

I shall record the following observations without offering for the present any interpretation of them. Within the last ten months the *right pupil was permanently distinctly larger than normal and responded sluggishly to light. An injection of adrenalin brought out a distinct constriction* which lasted about fifteen minutes. After the above mentioned stimulation of the cervical sympathetics, *the permanent dilation of the right pupil disappeared for about five weeks and an injection of adrenalin had no effect upon the pupil.* For the last three weeks the dilation of the right pupil had again returned.

On account of the very moderate effect which the intramuscular injection of adrenalin had caused in the left pupil in the last few days, an intravenous injection of adrenalin was tried on this animal for the first time. Not more than 0.3 c.c. of adrenalin (1 : 1000) were given through the ear vein. The right pupil remained unchanged, fairly dilated. *The left pupil became gradually dilated* so that after an hour the dilation was at the maximum. Half an hour later the animal fell over on its side, blood and foam escaping through the mouth and nose. The rabbit died of acute pulmonary edema.

At the autopsy, no sign of a ganglion could be discovered macroscopically on the left side; in the neighborhood of the seat of the ganglion the sympathetic nerve was lost in strands of connective tissue. (The abdominal aorta showed a few sclerotic patches.)

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<sup>1</sup> S. J. Meltzer and John Auer : *Journal of Experimental Medicine*, vii, 59, 1905.



## RESULTS OBTAINED BY THE INJECTION OF PLACENTA INTO ANIMALS OF THE SAME AND OF DIFFERENT SPECIES.

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Our anatomical knowledge of the various stages of pregnancy, except in its very earliest period, appears to be nearly complete. The clinical data dealing with the physiology and pathology of pregnancy and the puerperium are also numerous and well understood; but when we seek to obtain information about the agencies which govern the organism during the many changes incident to gestation, we meet with nothing but conjecture and hypotheses. The numerous, but often disconnected facts, upon which these various hypotheses have been founded, are based upon observations obtained at the bed side, the autopsy table, from chemical researches and animal experimentation. In view of these widely differing sources, it is not surprising that contradictions and gaps are numerous.

The object of this study has been to investigate experimentally whether actual proof can be found, that the chorionepithelium (the placenta) exerts a specific influence, whether trophic or other, upon the maternal organism. Two ways of demonstrating such an influence are at our disposal. The one consists of the injection of placental tissue into females of the same species, followed by examination of their generative organs, in order to ascertain whether any anatomical changes, particularly such as are found during gestation, have been produced. The other method is the injection of placental tissue into animals of the same and of other species with the object of producing a specific biological reaction, which can be demonstrated by various methods. In order to acquaint the reader with the current ideas entertained on this subject it will be necessary to refer to some of the literature.

It is generally conceded that the ovary instigates and regulates the periodical menstrual cycle, which is physiologically interrupted only during pregnancy and lactation until the woman reaches her menopause. According to the Born-Fränkell theory (1) which, however, from recent investigations must be accepted with some reserve, the corpus luteum exerts a powerful influence upon the nidation of the ovum. Halban (2) thinks it likely that the ovary and the corpus luteum elaborate products which are antagonistic to one another. When in the right proportion (this would be during gestation), each directly neutralizes the actions of the other. During this time, therefore, some other influence would have to bring about the numerous changes observed. Halban ascribes to the placenta this temporary governing or trophic function. Many observations have definitely shown that nervous influences play no rôle (section of the cord, lactation in transplanted breast tissue, etc.),<sup>1</sup> Halban has accumulated numerous clinical observations upon which he has built up an ingenious working theory. The placenta, according to this author, causes the hypertrophy and hyperemia of the uterus and of the neighboring organs. In response to the same stimulus the decidual reaction occurs in the uterine mucosa and sometimes in other tissues, and likewise occur the hyperemia and hypertrophy of the breasts. When the placental influence is removed, as after labor or abortion, the pelvic organs undergo a marked involution, whereas on the other hand the previously hypertrophied but inactive breasts begin to secrete. The so-called "menstruation" of the newborn, and the "milk" in the breasts of infants are also ascribed, by the same author, to the withdrawal of the placental influence. Many facts and interesting observations are adduced in this paper, but on the whole Halban's theory has not met with general acceptance. The sole experimental confirmation is that of Miss Lane-Claypon and Starling (3) who

<sup>1</sup> Eckhard, "Beiträge zur Anatomie und Physiologie," *Eckhard's Beiträge*, 1858, i, 1, sectioned the nerves leading to the breast in goats, and found that the secretion of the milk went on unchanged.

Ribbert, *Archiv für Entwicklungsmechanik*, 1898, vii, 688, transplanted the breasts of a young guinea-pig into subcutaneous pockets in the ears. During a subsequent pregnancy the transplanted organs were found to contain functioning acini.

found that the extract derived from a rabbit's placenta, or fetus, repeatedly injected into virgin rabbits produced an hypertrophy of the breasts.<sup>2</sup> The incomplete experiments I have performed along somewhat similar lines will be referred to later.

Another group of investigators have sought for the true explanation by means of anatomical investigation. Chief among them is Schmorl (4) who has with great regularity found trophoblastic elements in the maternal organs. Rarely in normal women, almost regularly in eclamptics, he has discovered emboli, composed of syncytium, in the blood vessels of the lung. Kollman (5) has shown that syncytium undergoes dissolution when subjected to the maternal tissue fluids.

A third group of investigations are based purely upon experimental work, seeking to apply the Ehrlich side-chain theory to solve the problem. The activity in this line of research is so great that new observations are constantly cropping up, and consequently older conceptions will require frequent revision or rejection. Some of the older work has been made doubtful by more recently acquired knowledge. Much of this work unfortunately is still quoted and used in support of theories and far reaching conclusions.

Basing their ideas on the observations of Schmorl and Kollmann, Scholten and Veit (6) set out to prove experimentally, that fetal syncytium is dissolved by the maternal organism. It had been known that the organism reacts to the injection of foreign cells by elaborating a cytolsin which brings about the disintegration of the invader. Animals (rabbits) were injected with human placenta; their serum dissolved placental cells in vitro. Liepmann, who will be referred to immediately, was unable to obtain similar results.

Kawasoye (7) found that human placental cells when placed in the serum of gravid women produced a slight cloudy precipitate and underwent a partial solution. His controls which were kept in sodium chloride solution were, however, quite inadequate.<sup>3</sup>

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<sup>2</sup> Lane-Clayton and Starling believe that the placenta and the fetus elaborate an *hormone* which produces the breast changes. They passed the fetal or placental maceration through a Berckfeld filter and injected the fluid.

<sup>3</sup> Kawasoye's experiments may be divided into three groups:

1. A placental antiserum was obtained by injecting human placenta into rabbits. Large placental injections caused death, small ones albuminuria; a

When it first became known that the injection of foreign (art-fremdes) proteid produced a reaction in the living organism and could be demonstrated by a visible precipitate, many investigations along these lines were undertaken. If proteid (serum, organs, red blood cells) of a certain species are repeatedly injected into an animal of another species, the serum of the immunized animal becomes cloudy when the serum or the organ extract of the animal used to produce the immunization is added in the proper proportion. The value of this reaction is generally accepted and it has great forensic importance [Uhlenhuth (8)]. It was not only thought, at first, that the reaction applied to the proteid of a given species, but it was believed that a given organ produced an even more specific serum. This refinement has, however, not stood the test of further research, and reaction only for species can in most instances be looked for [Rostoski (9), Sata (10)]. Liepmann (11) announced that rabbits injected with human placenta produced a serum, which contained precipitins for placenta or placental blood. If either of these tissues were added to the placental immune serum, a precipitate was formed. In a later communication (11) he acknowledged that the washed placenta used for immunization necessarily contained general body proteids (connective tissue, red blood cells) as well as the special placental cells, and that, to exclude a general "human reaction" a previous partial precipitation with the serum of a non-gravid subject and clearing by centrifugalizing was required, to exclude this source of error. Several other investigators [Weichardt (12), Opitz (13), Wormser (14)] have obtained negative results in repeating Liepmann's work; while Kawasoye and R. Freund (15) agree with his findings.

Veit (16) in an interesting monograph has based an elaborate theory which seeks to account for all the phenomena of pregnancy, tolerance could be obtained. Precipitine reaction was positive with the blood of grvida, with retroplacental and cord blood, and with placental extract but negative with blood from the non-gravid and males.

2. The blood of gravid women dissolved placenta in vitro.

3. Albuminous urine of gravid women also produced an antiserum when injected into rabbits. The action of this serum was weaker and less specific than that of placental antiserum.



both those in the mother as well as those of the fetus, largely upon the above quoted works of Scholten, Kawasoye, Liepmann, Kollmann, and Schmorl. The main conclusions, germane to this branch of the subject, are of such general interest that they cannot be omitted.

In broad outline Veit's explanation is as follows: Schmorl has demonstrated syncytium in the lungs of the mother. Liepmann has furnished the biological proof that syncytial constituents are present in the blood of pregnant women, and this fact substantiates Veit's assumption that the placental proteid follows the laws of antitoxin formation and conforms to the side chain theory. Kollman has shown anatomically that syncytium is dissolved by the maternal blood; this solution presupposes a solvent chemical substance in the blood—a lysin. Veit bases all that is to follow upon the assumption "*that there is a continued entrance of syncytium from the intervillous space into the veins of the mother, and a subsequent lysis of the syncytium*" from the very beginning of pregnancy to its termination. Veit's own investigations (17) have convinced him that purely physico-chemical processes (differences in osmotic pressure) do not account for the absorption of salts and proteids by the fetus. Absorption is attended to by the villi, which dip into the maternal blood and show a selective affinity for certain substances only. This selective activity has been demonstrated by Ascoli (18) who proved that egg albumen though present in the blood of the mother did not appear in the circulation of the fetus, and by Schenk (19) who noted quantitative differences in the hemolysins and agglutinins of mother and child.

A possible explanation, according to Veit, is that the nucleus of the syncytium may control the function of assimilation, and the cell protoplasm that of excretion. When the syncytial cell is filled with excretory substances it is cast off from the villus and falls a prey to solution in the maternal blood.

The absorption of iron, which is not in solution, but is an integral part of the hemoglobin molecule and is enclosed by the red blood cell, is brought about by a similar reaction. The lysin acts as an intermediary between the red blood cell and the syncytium, and when this action occurs in the intervillous space the erythro-

cyte is bound to the functioning syncytium of the villus. Thus hemoglobin is removed without producing general hemolysis. Hemoglobin has actually been demonstrated in the villi by Hofbauer (20).

Referring to the pathological phenomena of pregnancy Veit offers the following explanation: The albumin found in the urine of patients suffering from albuminuria of pregnancy, is a true heterologous albumen, such as Liepmann pronounced it to be; it is due to dissolved syncytium which has entered the maternal blood stream in large amounts. If the quantity is very great, the syncytium which is then only partly neutralized acts as a toxin on the kidneys, and produces an actual nephritis. The proof of the nature of this albumin lies in the fact that the specific precipitine reaction is obtained with such urines (Kawasoye). When the syncytium is in greatly excessive amount it no longer acts as a merely local poison, a renal irritant, but produces systemic symptoms which manifest themselves in the eclamptic seizure. Possibly other morbid conditions of pregnancy, such as hyperemesis, icterus, morbus maculatus, etc., are due to this same cause.

The views of Veit, as set forth in the article which has been mentioned, cover every branch of the subject, and in building up his hypothesis he has made use of practically all the work, which has been done along these lines. In the first place he has accepted the anatomical findings of Schmorl and Kollmann.<sup>4</sup> The discoveries of Schmorl may be considered conclusive.

The biological experiments, which are believed to show that the syncytium not only can, but does enter the maternal circulation, acts as a toxine on the tissues of the mother, and stimulates receptor formation, have not been convincing. In opposition to the positive findings of Veit and Scholten, Liepmann, and Kawasoye, Weichardt, Opitz and Wormser obtained negative results, as already stated. Veit has apparently not attached sufficient weight

<sup>4</sup>The work of Kollmann, as far as it bears upon this point, is not convincing. The material was obtained from a fetus three weeks old; some specimens were from pregnant monkeys. He interprets the vacuolization and disintegration noted in the syncytium, as due to lytic processes. It may be said, that changes, such as he pictures, are to be found in almost any rapidly multiplying tissue, and certainly do not necessarily imply any specific solvent process.

to the negative findings, particularly if we take into consideration the fact that some of the positive results were not constant or strictly specific.

Without entering into the merits of Veit's interesting hypothesis, which may yet prove to contain the true explanation of the phenomena of pregnancy, I have sought to repeat some of the work performed, and have used in part the methods already employed by others, in part newer or different methods.

1. To investigate Halban's placental theory three rabbits were repeatedly injected with fresh placenta of pregnant rabbits removed by operation just before term. These animals were used: (*a*) anatomically to study the uterus, ovary and tubes; (*b*) biologically to test the serum for precipitines. The difficulty in obtaining sufficient material for injection made it necessary to cut short this series of experiments somewhat summarily.

2. To control the work performed by Liepmann, etc., three groups of rabbits, each group composed of two animals, a male and a female, were injected repeatedly with the following materials: (*a*) A solution of nucleo-proteid obtained from the human placenta. This was tested for precipitines, and for deflection of complement by the method of Neisser and Sachs. (*b*) A maceration of human placenta made blood-free by washing in plain running water; it was tested by the same methods. (*c*) A maceration of human placenta made as bloodless as possible by washing with large quantities of normal saline solution; it was tested for precipitines, deflection, cytolysis, etc.

#### RABBITS INJECTED WITH PLACENTA OF RABBITS.<sup>5</sup>

A double purpose was kept in view in this series of experiments. Firstly, if placental tissue should prove to have an inherent specific action such as Halban ascribes to it, repeated intraperitoneal injections ought to produce distinct changes in the genital system. Such changes in the mammary glands were reported by Lane-Claypon and Starling a number of months after my experiments were

<sup>5</sup> This group of experiments was performed in the Pathological Laboratory of the College of Physicians and Surgeons during the months of April and May, 1906. I desire to express my obligations to Professor T. M. Prudden for the courtesies extended to me.

completed. The duration of pregnancy in the rabbit covers thirty days, therefore it would seem necessary to extend the injections over a period of about that length.

Secondly the use of the serum obtained, if potent, would avoid a disturbing factor not to be overcome if the injections were made into an animal of another species, namely hemolysins, and "a general species reaction," for the tissues of the animal used to produce immunization. The work of Kraus and Ludwig (21), and still more recently that of Schultz (22), shows that iso-hemolysins cannot be obtained in rabbits. It might be supposed that experimentally, at least, iso-syncytiolysins, would also not be formed. However the analogy is not quite exact, for in virgin animals (such as were used in this series) the placenta, if it differ biologically from other tissues of the body is a foreign substance. An article by Ed. Martin (23) has just appeared reporting attempts to prove the presence of iso-hemolysins and iso-agglutinins obtained in the following way. Twelve rabbits were operated upon, one half of the pregnant uterus (which in the rabbit is didelphous) being removed, and 0.5 grams of the mashed placenta at once injected into the ear vein of the same animal. In none of the experiments were either hemolysins or agglutinins obtained.

*Technic.*—Three young female rabbits were employed. A number of other rabbits were allowed to be impregnated at regular intervals, and the gravid uterus taken out at about the twenty-second to twenty-fifth day of gestation. The six to eight placenta obtained at each operation were at once cut up and mashed in a mortar, the maceration thinned out with an equal volume of normal salt solution and ten cubic centimeters injected intraperitoneally through a large needle into the experimental animals. Throughout, aseptic precautions were employed and no infection occurred. After each injection the animals did not take food for a few hours, but showed no other ill effects; however, during the entire course of the experiments they lost some weight. Nine injections were given to each rabbit at three-day intervals. On the fifth day after the last injection the animals were bled to death and the clear serum obtained in the usual way. The pelvic organs were at once placed in five per cent. formalin and prepared for examination by embedding in celloidin.

The material for histological examination consisted of blocks taken from at least four levels of the tubes and uterus, and longitudinal sections of each ovary. The controls consisted of material taken from normal animals killed in the laboratory during the time in which these experiments were performed. The result of

the examination of these organs can be summed up by stating that there was not the slightest difference between the organs of the injected and of the control animals. No decidual reaction, not even an increased vascularity, was found. Macroscopically no difference in size or appearance could be noted.

The serum tests were performed as follows:

Fresh serum from the injected rabbits was taken in increasing dilution (see Table), placed in small test tubes, and to this serum was added extract of rabbit's placenta (allowed to stand forty-eight hours and prepared in the cold) of various strengths. In another series a loop full of maceration of washed rabbit's placenta was added to the immune serum. Both series were at once brought to a uniform volume of two cubic centimeters by adding normal salt solution. They were placed in the thermostat for one hour and then in the ice-chest for from twelve to twenty-four hours. In none of these series did a precipitate appear.

Anti-serum.	Placental Extract or Placental Tissue.			
	0.1 c.c.	0.05 c.c.	0.01 c.c.	
0.2 c.c.	0.1 c.c.	0.05 c.c.	0.01 c.c.	
0.1	"	"	"	
0.05	"	"	"	
0.2	"	"	"	
—	"	"	"	

#### RABBITS INJECTED WITH HUMAN PLACENTA.<sup>6</sup>

*Placental Nucleo-Proteid.*—The work of Bierry and Meyer (24) and that of Beebe (25) and others lead one to expect that the nucleo-proteid, of a tissue, yields a more specific immune serum and gives less "general species reaction" than the serum obtained from injecting the washed organ. Levene (26) however says, that extracts made from various constituents of red blood cells and used for immunization, either give no hemolytic sera or sera far less potent than those obtained from the entire red blood cells. Pearce and Jackson (27) in a paper published recently, claim that a repetition of Beebe's work has given negative results. These investigators have made a very complete series of experiments from

<sup>6</sup> Almost all the human material used for these and the succeeding experiments was obtained through the kindness of Dr. George Ryder, resident obstetrician of the Sloane Maternity Hospital.

which they conclude that the nucleo-proteids act as mild toxic agents, affecting chiefly the excretory organs, and that they do not produce specific anti-sera. They say that Ehrlich and Morgenroth have shown that anti-sera are produced not by specific cells, but by specific free receptors. Specificity in the morphological sense cannot be demonstrated. The sole criticism that can be applied to the work of Pearce and Jackson is that they have prepared the nucleo-proteid by the "hot" method, which necessitates bringing the mashed organ to the boiling point! Such a method is inadvisable if biological reactions are to be employed. These authors did not attempt to make precipitine tests, but studied the effect of the injections upon the organs in vivo and also histologically.

*Technic.*—Many (ten to twelve) placenta were passed through a meat machine and washed in large quantities of 0.9 per cent. saline solution. The blood-free tissue, to which a 0.5 per cent. sodium carbonate solution in the proportion of 1:3 had been added, was placed in the ice chest for twelve hours. Microscopical examination of the last wash water still showed the presence of red blood cells in small number, about one hundred being present in the ordinary low power field. The extract was next filtered through several thicknesses of gauze and the nucleo-proteid precipitated by means of a slight excess of acetic acid. The precipitate was now washed by decantation and attempts made to redissolve it with sodium carbonate solution. It was found that unless the process was hastened, the carbonate solution did not act as a solvent, but a decinormal sodium hydrate caused solution. Reprecipitation and redissolving completed the process.

No exact analysis of the amount by weight represented by each unit of solution was determined, but as from twenty-five to thirty cubic centimeters of very concentrated solution were given, at each injection, a considerable quantity of the nucleo-proteid was used.<sup>1</sup> Dr. P. Levene of the Rockefeller Institute had the kindness to make a Phosphorus determination on the nucleo-proteid used in these experiments. The phosphorus percentage proved to be 0.35.

The solution was injected into two rabbits, a male and a female, intraperitoneally at intervals of about five days, seven injections in all being given to each animal. Serum was taken after the fifth injection and again after two additional injections. The animals flourished during the course of the experiments and gained in weight.

The serum of these rabbits was tested, while fresh, with placental extract (human placenta) and with bits of washed placenta, just as in the previous series. No precipitin reaction was obtained, nor did human blood-serum produce any result.

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<sup>1</sup> The first set of sera obtained from these animals were inactivated at 55° C. Noguchi's paper (*Journal of Experimental Medicine*, 1906, viii, 726) refers to the substances which he calls "protectines," or antilyssines, formed when sera are heated above 56° C. To avoid all possibility of error from this source the subsequently obtained sera were all inactivated at 52° C.

Another portion of each serum was inactivated at 55° C., for fifteen minutes and then tested by the method of Neisser and Sachs (28) for deflection of complement. This method is regarded as even more delicate than the precipitin reaction, although it is more troublesome and is somewhat capricious.

The rationale of the reaction is as follows: An indicator consisting of an independent hemolytic system is used. In my experiments rabbit's serum made lytic to hen's corpuscles by repeated injections of the washed corpuscles of hen's blood was employed. This anti-hen's serum was inactivated by heat. The other component of this series was hen's corpuscles diluted to a strength of five per cent. If fresh guinea-pig's serum (complement) is added, hemolysis is complete within one hour at a temperature of 37° C.

The second or main components of the reaction are the following: The placenta anti-serum (inactivated) is supposed to contain the amboceptor or specific binding-body if such is elaborated. The organ extract used for immunization must contain corresponding receptors; it is called the antigen. If to these two bodies combined in the proper proportion (see Table) a sufficient but not too great quantity of complement is added, the amboceptor should serve to bind the complement to the receptors. As the amboceptor is specific, it will serve only to bind complement to corresponding receptors. In other words, should an appropriate antiserum be obtained, the free complement is bound to the receptors, and when the hemolytic series (which contains no complement) is added, no hemolysis results. As actually performed, the amboceptor, antigen and complement are added in varying proportion, placed in the thermostat for one hour to allow deflection of complement to take place, and then the second series (hen's antiserum and hen's corpuscles) are added. The complete mixture is then again placed in the thermostat for two hours more. The result, *i. e.*, hemolysis complete, incomplete or absent, is read. The tubes are put in the ice chest for from twelve to twenty-four hours and the final reading taken.

In my experiments various quantities of the components were tried. The following table shows the proportion which was most economical of anti-serum and yet gave the greatest number of proportions of amboceptor and antigen. Throughout, just sufficient complement was used to assure complete hemolysis. The necessary quantity of complement was determined anew each time fresh complement was used.

To these and to all succeeding reactions enough normal salt solution was added to bring the total volume to four cubic centimeters.

When negative results were obtained in reactions similar to those described in the above tables, other experiments were performed, using as much as 1.0 c.c. of anti-serum, and 0.5 c.c. of placental extract. Only after repeated trials of these various proportions, was a negative result accepted.

Anti-serum (Amboceptor).	Placental Extract (Antigen).	G. P. Serum (Complement).	Hen's Anti-serum.	Hen's Corpuscles 5%.
0.2 c.c.	0.01 c.c.	0.04-0.025 c.c.	0.01 c.c.	1.0 c.c.
0.1	"	"	"	"
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
0.2	—	"	"	"
0.2	—	"	—	"
—	0.01	"	0.01	"
—	—	"	"	"
—	—	"	—	"
—	—	—	0.01	"
—	—	—	"	"
Placental Extract.	Anti-serum	G P Serum.	Hen's Anti-serum.	Hen's Corpuscles.
0.1 c.c.	0.1 c.c.	0.04-0.025 c.c.	0.01 c.c.	1.0 c.c.
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
—	"	"	"	"
—	"	"	—	"
0.1	"	"	0.01	"
0.1	—	—	0.01	"
—	—	0.04-0.025	"	"
—	—	—	"	"
—	—	—	"	"

Before proceeding to the complete reactions, which the tables show, it was necessary to determine in each case that the given anti-serum was inactivated and was neither in itself hemolytic or anti-hemolytic. Two of the anti-sera (those obtained from human placenta washed with normal salt solution) proved to contain a small trace of *amboceptor for hen's corpuscles*. This disturbing factor was removed by treating these sera, for one hour at 37° C., or over night at 0° C., with a great excess of hen's corpuscles and using the supernatant clear fluid after centrifuging. Various prepared extracts were tried. One set was obtained by placing the macerated washed placenta, diluted with normal salt solution 1:3 for forty-eight hours in a shaker, the other in the thermostat for forty-eight hours. A second set of extracts were made using plain distilled water. As no great differences were found, the extract finally used was the one prepared with salt solution in the thermostat. Toluol was added to prevent bacterial growth, and



this antiseptic was later removed by evaporation. The extract was always tested for its hemolytic action and a quantity far below this point used in the reactions.

The four nucleo-proteid sera obtained from the two injected animals showed no deflection of complement. In other terms, the presence of a specific antibody could not be demonstrated either by the precipitin or by the reaction for deflection of complement.

*Placenta Washed in Running Water.*—As not all the red blood cells could be removed by washing the placenta on a filter with normal salt solution, and it was not feasible to obtain the placentae so early that they could be exsanguinated by washing through the umbilical cord, the finely chopped placenta was placed in a large gauze bag and attached to the cold water faucet. By these means the tissue became practically blood free in a short time, the last wash water showing very few red blood cells, or rather "shadows." The drawbacks attached to this method were fully realized, but it was hoped to control the results by experiments which follow.<sup>8</sup> The placental tissue was then ground in a mortar with sand, and a thick, yet finely divided placental suspension prepared with sterile salt solution. Cultures of each batch of suspensions either showed no growth, or in the case in which growth occurred, an apparently non-pathogenic saprophyte was found.

Three rabbits, two males and one female, were used. One male died after the second injection, autopsy showing a peritonitis due to accidental perforation of the intestine. The other two animals received, respectively, six and eight injections intraperitoneally. The one which received eight injections was given 15 c.c. each time; the other was given from 25 to 30 c.c. The first animal gained over 200 grams in weight, the second lost nearly 400 grams.

The two sera obtained were tested both by the precipitin reaction and by the method of deflection of complement. *The results were absolutely negative.*

*Placenta Washed in Normal Salt Solution.*—The previous ex-

<sup>8</sup>Washing the tissue with a solution which was not isotonic made it not unlikely that at least some of the antigen would be carried away. Yet by this method a more complete removal of the red blood cells was obtained, and the microscopical examination of the tissue showed that enormous quantities of well preserved placental cells remained for injection.

periments, in which the placenta was rendered practically blood free, at the cost of losing at least some of its active constituents, resulted negatively. As a check and control, the placentae in the series now to be detailed, were first chopped fine with a meat machine, and then each placenta was washed on a filter with ten liters of salt solution, with constant stirring. A careful lookout was kept for all visible blood clots and these were removed. The last wash water appeared clear macroscopically, but under the microscope, from twenty-five to one hundred red blood cells to the low power field were noted. I have been unable to find any reference, in the literature, to the minimum quantity of blood necessary to produce an immune serum. The further treatment of the material in no way differed from that employed in the previous series.

*Technic.*—Three rabbits were used at the outset, two males and one female; but one male was so seriously injured in a fight with the others that the injections had to be discontinued.

Both the remaining rabbits received nine injections each, at intervals of about five days, from twenty-five to thirty cubic centimeters being introduced intraperitoneally. The animals were bled after the seventh and again after the ninth injection. Both gained markedly in weight during the treatment. In spite of the injections, the *female conceived and bore four normal young* during the course of the experiment. When bled to death she was found to be again pregnant (about twelve days gravid).

Of the four sera, the one serum obtained from the female (after the seventh injection) was rendered useless by being overheated, due to a faulty thermometer. The three other sera were tested for:

1. Precipitin reactions.

- a. Precipitin reactions with placental extract.
- b. Precipitin reactions with cord blood serum.
- c. Precipitin reactions with retro-placental blood serum.
- d. Precipitin reactions with normal blood serum from a male.
- e. Precipitin reactions with placental extract after saturation with male blood serum and with human red corpuscles.
- f. Precipitin reactions with urine (non-albuminous) of gravidæ.
- g. Precipitin reactions with urine (albuminous) of gravidæ.
- h. Precipitin reactions with urine of a male, containing albumin.

2. Reactions for deflection of complement.

- a. Reaction for deflection of complement with placental extract.
- b. Reaction for deflection of complement with normal human blood serum (male).

3. Agglutination reaction with human corpuscles.

4. Hemolysis reaction with human corpuscles.

5. Cytolytic reactions.

- a. Cytolytic reactions with emulsions of placental cells.
- b. Cytolytic reactions with small pieces of placental tissue.

The results obtained by the various tests follow, and as the three sera have given the same results, in approximately the same dilution, only one set of records will be described.

#### 1. PRECIPITIN TESTS.

- a. A faint but positive reaction was obtained with washed placental extract (0.05 c. c.) and the immune sera in dilutions of from 1:10 to 1:20 corresponding to 0.2 to 0.1 c.c. *Normal rabbit serum gave no precipitate to placental extract or to the other substances tested in Experiments e to h.*
- b. The cord blood, from two cases, gave similarly positive results.
- c. Retro-placental blood serum, from two cases, gave a distinct but weaker reaction.
- d. Normal human blood serum (from a male) gave a precipitate, in the same dilutions as in Experiment a.
- e. Partial precipitates were sought for. To the immune serum was added an excess of normal human serum. After the precipitate had formed, the fluid was cleared by centrifuging, and then placental extract added as in Experiment a. No precipitate appeared in the tubes to which placental extract had been added, nor in those into which more human serum was placed, although even stronger solutions than in Experiment a were used. After it was observed that the antisera were hemolytic, another method for obtaining a partial reaction was tried. An excess of a five per cent. solution of human corpuscles was added to the antisera and kept in the ice chest for twenty-four hours. To the clear solution was added placental extract and human serum, in the same proportions as in Experiment a. No precipitate was obtained. The control with more human blood cells showed no further hemolysis.
- f. The urines of six gravid women (containing no albumin) were tested, using 0.1 to 0.05 c.c. of urine, to the same quantity of antiserum as in the other experiments. No precipitate resulted.
- g. The urines of two gravid women (containing much albumin, one in the pre-eclamptic stage, the other toxemic) were tested. The one containing the most albumin gave a negative, the other a faint but positive reaction.
- h. The urine of a man containing much albumin gave a faint but positive reaction.

The precipitin reaction in all these experiments showed a faint but unmistakable *human reaction, but no specific placental reaction.*

#### 2. TESTS FOR DEFLECTION OF COMPLEMENT.

For experimental details see heading "Nucleo-Proteid Tests."

- a. For all three sera it was found that with from 0.004 to 0.001 c.c. of antiserum, and 0.01 c.c. of placental extract, using slightly more than the minimum amount of complement required (0.03 to 0.025 c.c.), hemolysis was incomplete or absent.

- b. With human serum (male) a positive reaction was obtained in much greater dilutions (down to 0.001 c.c. of human serum with 0.003 to 0.001 c.c. of antiserum).

Deflection of complement was more strongly marked in the case of normal blood serum than in that of placental extract.

3. AGGLUTINATION TESTS. Agglutination of a five per cent. solution of normal human blood corpuscles was obtained in dilutions of the antisera between 1:6 and 1:12, 0.5 c.c. of corpuscles being used. Normal rabbits' serum failed to agglutinate.
4. HEMOLYSIS TESTS. Hemolysis of 0.5 c.c. of a five per cent. solution of human corpuscles was obtained in dilutions of from 1:5 to 1:12 of the antisera, the controls with normal rabbits' serum again proving negative.
5. CYTOLYSIS TESTS.

- a. Suspensions of placental cells, obtained by the method used by Flexner and Noguchi (29) in their cytolytic experiments, were added to 0.5 c.c. of the undiluted antisera, to normal rabbits' serum and to normal sodium chloride solution, kept in the incubator at body temperature for one hour, and then in the ice chest for twelve hours. Unstained and stained specimens of the cells were examined. No very marked differences were noted. On the whole the specimens kept in normal rabbit serum showed the best preservation. In all the others the chromatin network was found more coarsely granular and the nuclear outline less distinct; while in the unincubated specimens, made for control, immediately after preparing the cell emulsion, there were numerous Langhans' cells in addition to the syncytium; in the incubated specimens the syncytial cell complexes greatly preponderated.

- b. A similar set of experiments to the foregoing were performed, using small pieces of placental tissue instead of isolated cells. All the specimens were transferred to a five per cent. solution of formalin embedded by the celloidin method, cut, and stained with hematoxylin and eosin. No differences were noted except that as in the previous series the specimens preserved in sodium chloride solution showed less perfect staining qualities. Otherwise the placental tissue was perfectly normal.

#### CONCLUSIONS.

The injection of rabbits' placenta into rabbits produces no isoprecipitins.

From the incomplete experiments performed it would appear that placental injections into animals of the same species cause no changes in the generative organs. Further research into this question will be pursued.

The injection of human placental nucleo-proteid, prepared from placental tissue made nearly blood free, does not produce an antiserum. This result confirms the conclusions of Pearce and Jack-

son, that nucleo-proteids act merely as mild toxic agents, without specific qualities.

The injection, into rabbits, of human placental tissue, rendered practically blood-free, fails to produce any specific reaction. This confirms the view that the serum reaction following the injection of cells into a foreign organism is largely due to the blood contained in the injected tissues.

The injection into rabbits, of the human placenta, made nearly blood-free, produces a weak "human reaction" which can be demonstrated by the reactions for precipitin, deflection of complement, agglutinin, and hemolysis. *No specific placental reaction can be shown.* This is in strict accord with the view that cytotoxines are not specific; that there is no morphological specificity.

The anti-sera obtained showed no cytolytic action; therefore no specific syncytiolytic action could be demonstrated.

If the information obtained in this investigation is applied to the theory of Halban, it will be noted that no experimental proof of the specific action of placental tissue upon the female generative organs could be demonstrated. The number of experiments performed, bearing upon this one point, were however too few to permit of a definite and final opinion.

The work dealing with Veit's ingenious hypothesis was more complete and carried out by many methods, which would necessarily act as a check upon one another. As the results of all these experiments were in complete harmony, I feel justified in making a positive statement that *no experimental proof of a specific placental immune reaction can be demonstrated by our present biological methods.* Whether Veit's hypothesis, thus deprived of its biological proof, must in consequence be discarded, is a question which I do not consider myself competent to answer.

In conclusion I desire to thank Dr. Simon Flexner, Director of the Rockefeller Institute for Medical Research, for extending to me the privileges of his laboratories. I also wish to acknowledge my indebtedness to Drs. Jobling, Noguchi and Levene for the frequent advice and assistance I have received from them.

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## THE NATURE OF THE ANTITETANIC ACTION OF EOSIN.

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In a previous publication<sup>1</sup> Dr. Flexner and I gave the results which we obtained from a study of experimental tetanus in rats and guinea pigs subjected to the influence of certain photodynamic anilines. To recall briefly the main facts brought out there, it may be stated that a solution of eosin, of a certain concentration, destroys in vitro the haemolytic and tetanospastic principles of tetanus toxin, and when applied to the site of inoculation of tetanus spores on threads, or tetanus-toxin, either entirely prevents the appearance of the tetanic symptoms, or delays the appearance and diminishes the effect, and, in rats, sometimes prevents the fatal issue. In a great number of instances in which the eosin is applied directly to the spore-infected area in rats, the lives of the animals can be saved. The next step in this investigation was the determination of the mode of action of the eosin through which the effects already described are produced.

The present paper deals with the manner of action of eosin upon *Bacillus tetani* without and within the body of animals. The subject falls naturally, therefore, into the two main divisions of the action of eosin upon the tetanus bacillus *in vitro* and *in vivo*.

### THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VITRO.

Relating to this topic, the following points were considered: (1) the influence of eosin upon the germination, growth, sporulation, morphology, and toxin production of *B. tetani*; (2) the bactericidal and sporicidal effects of eosin upon *B. tetani*; (3) the viability of *B. tetani* in eosinized media; (4) the toxin-producing power of *B. tetani* exposed to the action of eosin.

<sup>1</sup> Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

*Cultivation of Bacillus tetani in Eosinized Culture Media.* (a) *Eosinized Glucose Bouillon.*—Two kinds of eosin—"rein" and "gelb"—were employed in strength of 0.001 per cent., 0.01 per cent., 0.1 per cent., and 1 per cent. A vigorous culture of the tetanus bacillus was inoculated and incubated at 37° C. in an atmosphere of hydrogen for 10 days.

The control cultures were abundant; they contained typical bacilli forming few threads and showing few spores. The growth in 0.001 per cent. eosin was equal to the control, but microscopically the thread forms predominated and no spores whatever could be found. The growth in 0.01 per cent. eosin was equal to the control, but upon microscopic examination only asporogenous thread forms were seen. The growth in 0.1 per cent. eosin was slight and showed single, asporogenous bacilli. No growth whatever took place in the 1 per cent. eosin medium.

The first effect which the eosin produces, apparently, is to suppress sporulation of the bacilli, and the next is to increase thread formation. The limit of concentration of eosin which permits restricted germination is between 0.1 and 1 per cent., and is probably not far from the former concentration. A later determination placed this limit at 0.2 per cent. The cultures prepared as described were filtered through porcelain and tested for toxicity upon rats. The number of m. l. d. per cubic centimeter of filtrate was as follows:

Control	= 1000 m.l.d.
0.001 per cent. eosin	= 700 m.l.d.
0.01 per cent. eosin	= 100 m.l.d.
0.1 per cent. eosin	= 1 m.l.d.
1.0 per cent. eosin	= 0 m.l.d.

No remarkable difference in the properties of the two kinds of dye was noticed. The reduction in toxicity is greater than the reduction in growth caused by the eosin. The diminished toxicity has, doubtless, a two-fold origin: it arises from the diminished multiplication of the bacillus, and from the injury exerted by the eosin upon the toxin originally produced. The degree of this destruction can be deduced from the relative strengths of the filtrates from the control and the 0.001 per cent. eosin cultures, and it amounts to about 30 per cent. As the eosin strength grows, the effects upon the multiplication and toxicity increase, but not in equal ratio. A corresponding comparison of the hæmolysin (tetan.



olysin) present in the several filtrates was also made. Each cubic centimeter contained the following units:

Control filtrate contained			*C.H.D.	20	*M.H.D.	333
0.001 per cent.	"	"rein"	"	12	"	242
0.001	"	"gelb"	"	10	"	200
0.01	"	"rein"	"	4	"	66
0.01	"	"gelb"	"	3	"	50
0.1	"	"rein"	"	0	"	0
0.1	"	"gelb"	"	0	"	0

\* C.H.D. = complete hæmolytic dose; M.H.D. = minimal (trace) hæmolytic dose.

Eosin destroys or suppresses the tetanolysin in the cultures as it does the tetanospasmin, a fact which could be predicted on the basis of the observations of Flexner and Noguchi.<sup>2</sup> Note should be taken of the greater activity in this respect of eosin "gelb" as compared with eosin "rein."

(b) *Eosinized Glucose Agar*.—The advantage for the study of *B. tetani* which glucose agar has over glucose bouillon is derived from the fact that in the former medium sporulation is much more abundant, and is already easily noticeable after twenty-four hours growth. At the end of seven days spores are numerous, and at the expiration of about thirty days, no vegetative bacilli remain. Hence, to glucose agar the following strengths of both eosins were added, 0.01 per cent., 0.02 per cent., 0.05 per cent., 0.1 per cent., 0.2 per cent., 0.5 per cent., and 1 per cent. Stab inoculations were made; cultivation at 37° C.

The growths were well-marked in the tubes below 0.05 per cent. strength; above this limit the growths diminished progressively with increase in concentration. On microscopical examination, it was found that 0.1 per cent. of eosin was the limit of concentration for spore formation. Below this concentration, sporulation took place; at and above it, no sporulation occurred even after three months. The number of spores in eosinized media is below the number in ordinary media; the situation of the spores in the former media is the centre of the body. Transplantation of the atypical sporulating bacilli to simple glucose agar brings about immediate

<sup>2</sup> *Op. cit.*

return to typical mode of sporulation. The limit of germination is for eosin "rein" about 0.5 per cent., while for complete germination, it is about 0.2 per cent. Strengths above 0.5 per cent. inhibit vegetation of spores, but they do not kill the spores. Eosin "gelb" is somewhat more inhibitory. Concentrations above 0.02 per cent. prevent sporulation, but permit vegetation; above 0.5 per cent. no vegetation occurs.

(c) *Eosinized Tissue Bouillon*.<sup>3</sup>—The tetanus bacillus grown aerobically at 37° C. in tissue bouillon produces abundant spores in a few days. The sediment which forms in the cultures in a few days, is composed largely of spores. The effect of eosin "gelb" on this process was studied by adding strengths of 0.001 per cent., 0.003 per cent., 0.01 per cent., 0.03 per cent., 0.1 per cent., 0.3 per cent., 1 per cent., and 2 per cent. of the dye. The tissue absorbs a part of the dye; the cultures were incubated at 37° C. for twenty hours when first examined, and subsequently for twenty-nine days longer.

After 20 Hours.		
Control.	Good growth; gas formation.	C.S.* single bacilli; no spores.
Eosin "gelb" 0.001 %	Idem.	C.S. chiefly single, few threads, no spores.
" " 0.003	Idem.	C.S. bacilli chiefly in chains, no spores.
" " 0.01	Less growth.	C.S. long threads only showing vacuoles and irregular contours.
" " 0.03	Idem.	Idem.
" " 0.1	Slight growth.	C.S. very few single bacilli, small number of spores.
" " 0.3	Doubtful growth.	C.S. few bacilli and spores.
" " 1	No growth.	C.S. very few spores.
" " 2	Idem.	Idem.
After 30 Days.		
Control.	Fluid clear; deposit heavy.	C.S. all bacilli bear spores.
Eosin "gelb" 0.001 %	Idem.	C.S. numerous threads and spores.
" " 0.003	Idem.	C.S. nearly exclusively in threads; very few spores.
" " 0.01	Idem.	C.S. threads; no spores.
" " 0.03	Idem.	Idem.
" " 0.1	Slight deposit.	C.S. a few short chains; many single bacilli; few spores.
" " 0.3	No change.	
" " 1	No change.	
" " 2	No change.	

\* C.S. indicates cover slip preparations stained in the usual way.

<sup>3</sup> Theobald Smith, *Jour. of the Boston Soc. of the Med. Sciences*, 1901, iii, 340.  
Tarozi, *Centralbl. f. Bakt.*, etc., Orig., 1905, xxxviii, 619.

The foregoing observations are in conformity with those already recorded. In spite of the vigorous growth of the bacilli in this medium containing small quantities of eosin, the biology of the organisms is influenced directly as regards their power to segment and to form spores. With increasing concentrations of the eosin these effects become more pronounced, until in the concentration of 0.1 per cent. only part of the transplanted spores are enabled to germinate, and the vegetative bacilli produced by this imperfect germination are restrained from free multiplication. This process of imperfect vegetation comes, I believe, to play a very interesting part in developing immunity in inoculated rats treated with eosin, of which phenomenon I will have occasion to speak hereafter.

(d) *Bactericidal and Sporocidal Properties of Eosin upon B. tetani*.—Glucose bouillon cultures of the tetanus bacillus do not form spores within forty-eight hours, hence such cultures can be employed to test the bactericidal effect of strong solutions of eosin. It was found that such spore-free cultures when mixed with eosin to the concentration of 2 per cent., and kept for fifteen minutes in diffuse light, fail to grow upon replantation. If the eosin strength falls below 1 per cent., not all the bacilli are killed. Contact for twenty-four hours of vegetative sporeless bacilli with eosin in solutions above 0.1 per cent., causes their death. The bactericidal effect of eosin is increased by exposure to the sun: eosin in a strength of 0.02 per cent. can cause in eight hours the death of vegetating bacilli, when exposed directly to the sun's rays. If the experiments are made under anaerobic conditions the results are not essentially different.

The spores of the tetanus bacillus display far greater resistance. Solutions of eosin of 5 per cent. and 0.05 per cent. failed to bring about their destruction after exposure under aerobic and anaerobic conditions to the direct rays of the sun for thirty hours.

(e) *Viability of B. tetani in Eosinized Media*.—Cultures eighty-eight days old of the tetanus bacillus in glucose agar containing 0.1 per cent. eosin "gelb" showed no spores. On transplanting from these cultures into glucose agar, a feeble growth containing few spores after seven days was secured. Transplantations into eosin-free media from this slight growth gave cultures which be-

haved, in all respects, in a normal manner. From this experiment the conclusion can be drawn that concentrations of eosin which are not quickly fatal to sporeless tetanus bacilli reduce in the first generation their power of reproduction but do not suffice to kill them outright even after long periods of contact.

(f) *Can B. tetanus be Rendered Durably Asporogenous by Eosin?*—Eosin in the strength of 0.01 per cent. reduces spore-formation in *B. tetani* and in the strength of 0.1 per cent. prevents it entirely. A strain of *B. tetanus* was cultivated in 0.01 per cent. and 0.1 per cent. successively through many generations. At each transfer to eosinized medium a control culture was made in glucose-agar to observe the point of final disappearance of the spore-bearing faculty. In the case of eosin "rein," the bacillus withstood fairly well the successive implantations into the eosinized medium, but the faculty to produce spores in ordinary media was not lost after eight generations, covering a period of three months, in eosinized glucose agar. A reduction of the spore-formation was noted in the first generations in the plain glucose medium. In the case of eosin "gelb," the bacillus survived for three generations only in 0.1 per cent. eosin-glucose-agar, and for the eight generations in the 0.01 per cent. medium. No permanent alteration of spore-bearing capacity was effected in the latter cultures.

(g) *Is the Toxin Producing Power of B. tetani Affected by its Growth in Eosinized Media?*—Two cultures of *B. tetani* were kept in 0.1 per cent. eosin "gelb" glucose-agar for eighty-eight days, after which they were renewed by transplantation to glucose bouillon. After forty-eight hours growth at 37° C., these cultures and suitable control cultures were filtered through porcelain. The toxicity of the filtrates was approximately equal. The deduction from this experiment is obvious: no permanent influence upon the toxin-producing faculty of *B. tetani* is exerted by long contact of eosin in concentrations below the bactericidal limit.

#### THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VIVO.

The next subject of the study taken up related to the manner in which eosin acts in preventing tetanus in rats inoculated with tetanus bacilli or their spores.<sup>4</sup> The effects to be explained are,

<sup>4</sup> Flexner and Noguchi, *op. cit.*

briefly, these: rats, beneath the skin of which tetanus spore-threads are placed, regularly develop tetanus and die. If, however, the inoculated rats are treated by injections of eosin about the spore-thread, many recover, and some even fail to develop any symptoms of tetanus. Injection of the eosin in other parts of the body may delay the appearance of tetanus and the fatal issue, but does not suffice wholly to prevent them. The effect of the eosin on the local reaction to the tetanus bacilli in the inoculated rats is to be explained.

*Fate of Spores Introduced into the Body on Threads.*—Eighteen rats (weighing about 90 grams each) were inoculated beneath the skin of the thigh with spore-threads of *B. tetani*, free from toxin, on June 8, 1906. The six of these left untreated (controls) developed tetanus in two to six days time (average, three days). Death usually resulted on the third day after the appearance of the tetanus. Cultures were made from the threads and the liver.<sup>5</sup> Tetanus bacilli were recovered from the threads in all six animals and from the liver in three animals. The remaining twelve rats were treated with eosin "rein." Doses of 0.2 to 0.4 c.c. of a 2 per cent. solution were injected successively on three days, beginning immediately after the inoculation, about the thread, and intermittently (every other day) for three more injections. The treated animals did not develop tetanus, and a part of them were alive and healthy four months after the inoculation.

The first question which I asked myself is, *Do the tetanus spores germinate and multiply under the influence of the eosin, and what becomes of the germinated bacilli?*

*Experiment. Rat No. 7.* A part of the spore-thread was removed forty-eight hours after inoculation and during the eosin treatment. Cover slips prepared from it showed many spores, a small number of bacilli and a few leucocytes. A second portion of the thread was removed on the ninth day (day of last eosin injection). Cover slips showed very few bacilli and many spores and leucocytes. Cultures gave pure growths of *B. tetani*. On the thirteenth day, the remainder of the thread was removed. Now only spores could be found, and leucocytes were no longer present. The rat remained well.

*Rat No. 8.* Same treatment; examination on fifth day; among many spores a small number of bacilli—vegetative forms—were seen.

<sup>5</sup> Tarozzi, *Centralbl. f. Bakt.*, etc., 1906, xl, 305, 451.

The study of the spore threads in the two inoculated rats show conclusively that the injections of eosin do not wholly prevent the germination of the spores, but that the germination is largely suppressed; and it also renders the view very probable that of the germinated spores few or none multiply in the eosinized tissues, while after a short time (few days) the vegetated bacilli themselves disappear. Attention is called to the observation that the tetanus spores remain in an intact state for many days in the inoculated region of the body.

The next question which I asked myself is, *What becomes eventually of the ungerminated spores, and, supposing they remain locally in the tissue, do they suffer any alteration in toxin-producing power?*

*Experiment. Rat No. 9.* Inoculated June 8. Eosin-treated. No symptoms, June 24. Removed the spore-thread which was found to be surrounded by fibrous tissue and adherent to the fascia. Eosin had disappeared. Cover slips showed many spores and no bacilli. A pure culture of *B. tetani* obtained in tissue bouillon.

*Rat No. 10.* The thread was removed nineteen days after inoculation. The result was in all respects like that of rat No. 9. Toxin of the usual strength was yielded by the cultures.

*Rat No. 11.* A part of the spore-thread was removed twenty-three days after the inoculation. Cover slips showed many spores which yielded a pure culture of *B. tetani* containing a strong toxin. The second wound healed without giving rise to tetanus. Animal remained well.

By these observations it is shown that the healing of the local wound containing the eosinized threads, proceeds in a manner similar to that of a sterile wound; and it is also shown that, by virtue of the eosin treatment, the tetanus spores are reduced to the value of innocuous foreign bodies. In the course of this process, the tetanus spores, already quiescent, remain in the healed tissues for an indefinite time. They are not devitalized by the tissues, or, apparently, altered in any essential way, at least, their toxin-producing power is not impaired by the new conditions under which they survive.

If the results obtained in vitro and in vivo are compared certain correspondences, as well as certain differences, can be noted. Perhaps the most important difference consists in the disappearance of the vegetative bacilli in the body, and their persistence in the test tubes, in the tests with eosin; and the germination of the re-

strained spores in the culture once the eosin is no longer present, while, in the body, the tissues already advanced in healing restrain the vegetative propensities of the spores, although the toxin has been entirely eliminated.

The healing of the wound of the second operation in rat No. 11, without giving rise to tetanus, all the eosin having meanwhile disappeared, is an interesting fact, and one that deserves further consideration. The cultivation experiment proved that living tetanus spores, capable of toxin-production remained in the thread; and yet the remainder of the thread, in the tissues, gave rise to no symptoms. It might be thought indeed, that the spore threads, after this long sojourn in the body, are not capable of directly infecting a second animal. A test of this possibility was easily made.

*Experiment. Rat No. 12.* A normal rat was inoculated on July 9 beneath the skin of the thigh with a spore-thread removed on the thirty-second day from an eosin-treated rat. On July 11 the animal was tetanic, and on July 13 it was dead. A pure culture of *B. tetani* was recovered from the thread.

The apparent innocuousness of the second operation for the removal of spore-threads in animals treated long before with eosin, and the great susceptibility of the normal rat to the threads after this long residence in the body, brought up the question of the possible existence of a local immunity to tetanus in the eosin-treated animals. This idea was, of course, capable of experimental verification. The experiments relating to it form the subject of a separate publication.

#### SUMMARY.

Eosin, if present in cultures containing tetanus spores, prevents the germination of these spores when its concentration (in glucose bouillon) reaches 0.2 per cent. When the concentration of the eosin sinks to 0.01 per cent., germination of the spores is no longer inhibited, but the vegetative bacilli developed from the spores execute a highly restrained form of multiplication. When the eosin concentration sinks to 0.001 per cent., vegetation and multiplication of the bacilli become more active, but no new spores are formed even after long periods of time. With glucose agar it is not until the concentration of the eosin in the cultures falls to 0.05 per cent. that sporulation again appears. At this concentration of the eosin,

very few spores are formed; but as the eosin sinks lower and lower, sporulation becomes more active, until with 0.001 per cent. it is essentially of normal degree. In concentrations of 0.003 per cent., eosin prevents perfect segmentation of the multiplying bacilli, with the result that, finally, long and convoluted threads of bacilli are produced. The spores which are formed in a medium containing 0.01 per cent. of eosin are often situated at the centre and not at one pole of the bacilli.

Eosin in a strength of 2 per cent. is capable of destroying the vegetative bacilli, if the contact is prolonged to fifteen minutes, and in strength of 0.1 per cent., in twenty-four hours. Placing this latter mixture of bacilli and eosin in the sunlight greatly hastens the bactericidal effect, and the bacilli are found to be incapable of growth at the end of several hours. Eosin in high concentrations is not capable of killing the tetanus spores, even after long exposure to sunlight (thirty hours).

The toxin production of tetanus bacilli grown in eosinized culture media diminishes as the concentration of the eosin increases. This effect is brought about partly by the restraining action of the dye on vegetation, and partly by its detoxicating action upon the poison.

The toxin-producing power and the virulence of tetanus bacilli are not permanently modified by contact with eosin for a long period, or by successive cultivations in eosinized media.

Eosin is likewise capable of restraining the vegetation of tetanus spores in the animal body. In spore threads inserted beneath the skin of rats, and surrounded with eosin in solution, a very restricted vegetation takes place. If the injections of eosin are repeated, vegetation soon ceases and the vegetated bacilli degenerate and disappear.

The ungerminated tetanus spores remain alive in a latent condition indefinitely in the healed wound beneath the skin. These spores do not lose power to grow outside the body, or inside the body of animals under favorable conditions, or to produce toxin in a characteristic manner.



## LOCAL IMMUNITY TO TETANUS IN INOCULATED RATS TREATED WITH EOSIN.

By HIDEYO NOGUCHI, M.D.

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Previous studies having shown the mechanism of the immunity to tetanus which develops in rats treated locally with eosin, my attention was directed by Dr. Flexner to certain indications of the occurrence in the treated animals of a local in contrast to a general immunity to the tetanus bacillus. Our studies<sup>1</sup> had already shown that the spores on silk threads in the healed eosin-treated wounds remain alive for an indefinite period, and the threads removed and transplanted to other rats, or to the opposite side of the body of the same rat, caused fatal tetanus. And yet, the operation for the removal of part of the spore-threads was not followed, in spite of the trauma inflicted and the portion of the spore-thread which remained, by any tetanus in the extremity originally infected. Since it appeared that the conditions were, after the second operation, similar on both sides of the body, or in the two animals, an explanation of the different reactions observed was required. It seemed natural to assume, for the sake of further experiment, that the restricted germination and vegetation which take place in the eosin-treated animals produce a small quantity of tetanus-toxin, which, acting locally upon the tissues, gives rise in them to a degree of immunity to the action of the tetanus poison.<sup>2</sup>

The observations upon which this hypothesis was first based will be given; but it should be stated in advance that the tetanus-bearing threads were introduced subcutaneously in the thigh and not deeply into the tissues. After the healing process is complete, the threads come to lie in a small mass of scar tissue between the superficial

<sup>1</sup> Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

<sup>2</sup> Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

fascia and the skin. Except for the cicatrix the tissues all return apparently to the normal condition.

*Experiment I.*—In order to determine whether the spores remained alive in the inoculated rats after the healing process was complete, a portion of a thread was removed thirty days after inoculation and fifteen days after the cessation of the eosin treatment; no trace of the eosin remained at this time in the tissues. The removed portion of the thread was used for preparing cultures and for inoculating rat No. 12. Tetanus bacilli grew in the cultures. Tetanus developed in rat No. 12 on the second day, and death from tetanus took place on the fourth day after inoculation. The treated and operated rat remained well.

Before discussing further the significance of this experiment, a selected series of protocols bearing on this question of local immunity to tetanus will be first given.

*Experiment II. Rat No. 13.*—Thirty days after inoculation and fifteen days after cessation of eosin treatment, a portion of the healed-in spore-thread was removed from the right thigh. No tetanus followed the trauma, and the wound quickly healed. Six days later, a second portion of the thread was removed and implanted subcutaneously under the skin of the left thigh. Tetanus developed on the left (new) side on the second day, and death occurred on the fourth day after this inoculation. The right (original) hind leg remained entirely free from tetanus.

*Experiment III. Rat No. 14.*—Eosin treatment. Seventeen days after inoculation the thread was removed from the right thigh; the wound of the second operation was completely healed in seven days, during which period no tetanus developed. July 12, reinoculation of spore-threads beneath the skin of both thighs. July 16, left leg tetanic; right leg free. July 18, tetanus has progressed on left side; right side free. July 19, rat dead. The animal lived three days after the first appearance of tetanus. The right (old inoculated) leg remained free from tetanus until the end.

*Experiment IV. Rat No. 15.*—Spore-thread inoculation into right leg June 8; eosin treatment until June 20. No tetanus. Spore-thread removed July 28; no tetanus. October 9 (123 days after inoculation, and 111 days after last injection of eosin), 0.00008 c.c. tetanus toxin injected into each thigh (0.0003 = m.l.d. in three days). Tetanus developed in the left leg in forty hours, and advanced rapidly; slight tetanus developed in the right leg after three days, and advanced slowly. Death on seventh day.

*Experiment V. Rat No. 16.*—Spore-thread inoculation into right leg October 29; eosin rein treatment until November 8. No tetanus. December 1 (33 days after inoculation and 25 days after last injection of eosin) 0.0004 c.c. tetanus toxin injected into each thigh. Tetanus developed in left leg after three days, and advanced rapidly; a mild form of tetanus developed in the right leg and advanced slowly. Both sides became strongly contracted; death on sixteenth day.

*Experiment VI. Rat No. 17.*—Spore-thread inoculation into right thigh October 19. Eosin gelb injection until November 2. No tetanus developed. December 1 (43 days after inoculation and 29 days after last injection of eosin),

0.0004 c.c. of tetanus toxin injected into each thigh. No tetanus developed in five days. Second injection into each thigh of 0.0004 c.c. toxin. Next day tetanus began in left leg; none in right leg. The tetanus advanced in the left and remained absent from the right side until death, which took place on the ninth day.

Experiment VI brings out clearly, for the first time, the existence of a degree of general immunity to the toxin which is, however, able to off-set a small and limited amount only of the effects of the toxin. The local immunity of the right thigh proved, upon a second injection of the toxin, to be still effective, while the general immunity, through which the left leg had been protected from the consequences of the first injection, was exhausted by it. The next experiment indicates that even a higher degree of general immunity may arise from the local infection.

*Experiment VII. Rat No. 18.*—Spore-thread inoculation into right thigh; eosin treatment. No tetanus developed. October 9 (123 days after inoculation and 111 days after last injection of eosin), 0.00008 c.c. tetanus toxin (0.00008 = m.l.d.) injected into each thigh. No tetanus after three days. October 12, 0.00004 c.c. toxin injected into each leg. October 19, no tetanus developed. The spore-thread from this animal transplanted to another rat produced typical tetanic symptoms, followed by death in two and a half days.

Experiment VII is a stronger confirmation even than experiment VI of the idea that general immunity to tetanus toxin may be developed in the eosin-treated rats. The degree of general immunity is, in almost all the rats, lower than that of the local immunity. Rat No. 18 seems to supply the one exception to what appeared as the rule in this respect. It was now desirable to ascertain by a direct experiment whether the blood of the eosin-treated rats contained a measurable quantity of tetanus antitoxin.

Three series of rats were taken: (1) normal rats as controls; (2) rats which after the spore-thread insertion had been treated with eosin "gelb;" (3) rats which after inoculation had been treated with eosin "rein." The rats were bled from the carotid artery, and the serum collected. On account of the small amount of blood yielded by these animals, the blood of the several animals of each series was collected together. The eosin-treated rats were bled from sixty-four to seventy-four days after the cessation of the eosin treatment. The following tabulation gives the results of the tests. In each instance, the mixtures of serum and tetanus

toxin were kept at 37° C. for half an hour before injection. The injections were made beneath the skin of the thigh.

Serum Injected.	Toxin Injected.	Tetanus Developed.	Result.
Normal 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Normal 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 6th day.
Saline 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Saline 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 5th day.
Eosin gelb "a" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus increased but did not extend beyond injected leg. 28 days after injection still alive; tetanus gradually disappearing.
Eosin gelb "b" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus progressed, death on 9th day.
Eosin rein 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Death on 10th day.
Eosin rein 0.3 c.c.	0.002 c.c. (2 m.l.d.)	5th day.	Death on 15th day.

The experiments which I have set down in this article seem to me to afford convincing proof of the existence of local immunity to tetanus infection and tetanus intoxication. The observation is, I believe, new and not without interest in its bearing on the theory of the "building sites" of immune bodies in the animal organism. Examinations of the lesion consisting of the scar enclosing the spore-threads have shown it to be superficial, limited to the fascia and adjacent subcutaneous tissue, and to measure a few millimeters in extent only. There are no anatomical changes discoverable which would connect the lesion with the deeper muscles, fascia, nerves, etc.

From the studies made of the spore-threads at different periods during the eosin treatment,<sup>3</sup> a complete idea of the evolutions through which the introduced spores pass has been obtained. A certain small number of the spores germinate and grow into vegetative bacilli, which, after having reached this stage of development, remain as such for a time, or, possibly, pass through a small series of divisions with the production of a few generations of bacilli. The number of divisions is at best few, and the total number of bacilli produced from the spores is relatively small. The vegetative bacilli degenerate within a few days, and the majority of

<sup>3</sup> Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

the spores do not germinate at all, but remaining alive and quiescent, are enclosed in the scar tissue.

Doubtless, there is associated with the low degree of vegetation of the tetanus bacilli toxin production. This toxin, always small in amount, is held chiefly in or near the locality in which it is formed by the surrounding wall of eosin through which it must, in the first place, pass slowly and with difficulty, and which, in the next place, would tend to detoxicate it on its passage. The effect, therefore, of the toxin would be exerted locally, if anywhere, and if any cells capable of being excited to antibody-production exist in the situation in which the toxin finds itself, they, presumably would be stimulated into activity. Should any of the toxin, in a still active state, diffuse beyond the eosin barrier, it would find itself in places from which it could readily pass into the blood and into the internal organs, where it would be exposed to the usual conditions of antibody formation. I conceive that the mechanism of the antibody production in the particular case brought out by these experiments is to be explained somewhat in the manner of this supposition.

The questions immediately arise as to which cells in the local tissues are concerned with the elaboration of the antitoxin, and whether the antitoxin as such is stored in these cells for immediate liberation when called forth by the presence of the specific antigen. As regards the first question, no definitive answer can be given. From the circumstances of the experiment, I am led to suppose the connective tissue cells, possibly the endothelium of the lymphatics as well, as the cells yielding the antibody. The extremely superficial character of the lesions must be kept in mind in attempting to fix the parts the cells of which participate actively in the production of this local immunity. But I cannot bring forth proof that the active cells are so strictly limited and so sharply circumscribed as I have indicated. It would, theoretically, seem to be less difficult to supply an answer to the second question. It does not seem to me highly likely that so diffusible a substance as antitoxin in the free state could remain in a state of high concentration through many months in one part of the body at the same time that it existed in very low concentration elsewhere in the body.

On the contrary, I should be more inclined to the view that a large part of the general antitoxic immunity which the rats exhibited may have been derived from the antibody absorbed from its local site of production. The cells of the locally immune tissues have, I assume, undergone a physiological change which endures for many months, at least, enabling them to withstand the injurious effects of tetanus bacilli and tetanus toxin either by means of rapid liberation of antitoxic substances, or by an increased form of resistance to and destruction of poison (*Giftfestigkeit*) and bacilli with which the liberation of antagonistic antibodies is not necessarily associated. Upon what remarkable changes in function or structure this power depends, we are in total ignorance, and any speculation must, therefore be wholly hypothetical.

The literature on immunity contains, as is well-known, other examples of local immunity. Doubtless in every case, at some period of antibody production, there exists a local immunity which exceeds in degree that which the blood is able to display. This fact must be acknowledged by all who believe antibodies not to be formed chiefly in the blood itself. It will suffice merely to refer to the observations of Pfeiffer and Marx<sup>4</sup> on the organs in which the antibodies to the cholera bacillus are produced; to the experiments of Wassermann and Takaki<sup>5</sup> of the fixation of tetanus toxin by the brain and other organic tissues; to the studies of Wassermann and Citron<sup>6</sup> on the local production by the pleural and peritoneal endothelium of antibodies (bacteriolytic, possibly others also) for the typhoid bacillus; to the interesting experiment of von Dungern<sup>7</sup> on the local production of precipitin for Maja serum in the anterior chamber of the eye; and, finally, to the observations of ophthalmologists<sup>8</sup> upon the local immunity developed by the conjunctiva to abrin inoculation, a phenomenon which Römer studied with great care and precision. Theoretically, there is much similarity in all these observations, since they prove that anti-

<sup>4</sup> *Zeit. f. Hygiene und Infektionskrank.*, 1898, xxvii, 272.

<sup>5</sup> *Berl. klin. Woch.*, 1898, xxxv, 5.

<sup>6</sup> *Zeit. f. Hygiene und Infektionskrank.*, 1905, I, 331.

<sup>7</sup> *Die Antikörper*, Jena, 1903.

<sup>8</sup> Von Hippel, *Archiv f. Ophthalmolog.*, 1883, xxix, 213. Sattler, *Klin. Monatsbl. f. Augenheilk.*, 1883, xxi, 207. Neisser, *Fortsch. d. Medicine*, 1884, ii, 73. Römer, *Archiv f. Ophthalmologic*, 1901, lii, 172.

bodies are produced locally. The gradually increasing number of facts relating to this subject tend to exalt in importance cells which hitherto have been regarded as indifferent in respect to antibody production: namely, the cells of the connective tissues, lymph spaces, lymph vessels and serous cavities. The particular observations which form the basis of this paper show that the antibodies to the tetanus poison can be produced in quantity by other cells than those of the central nervous system, for which, apparently, tetanus toxin has an especial affinity.





## PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF MAGNESIUM SALTS.<sup>1</sup>

### V. THE INFLUENCE OF NEPHRECTOMY UPON THEIR TOXICITY.

BY S. J. MELTZER AND D. R. LUCAS.

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*Introductory.*—The narcotic effect as well as the toxicity of magnesium salts, when introduced subcutaneously, depend to a great degree upon the readiness with which they are eliminated from the body. By what path are these salts eliminated? Are they excreted by the intestinal canal or are they eliminated through the kidneys? Meltzer and Auer<sup>2</sup> state that from their experiments they gained the impression "that animals which urinated frequently had a better chance for recovery. Early urination seemed also to prevent the full development of anesthesia. Furthermore it seems that magnesium sulphate after subcutaneous injection acted as a diuretic." Such observations seemed to indicate that the kidneys are taking an essential share in the elimination of the magnesium salts. On the other hand J. B. MacCallum<sup>3</sup> states that magnesium salts (magnesium chloride) not only do not act as diuretics, but exert directly an inhibitory effect upon the urinary secretion. Furthermore from a remark recently made by Mendel it seems to follow that magnesium as an alkali earth leaves the body by the bowel. Mendel<sup>4</sup> states that the studies of the paths of excretion for inorganic compounds seem to "indicate a noteworthy difference in the mode of elimination of these alkali metals in contrast with corresponding salts of the alkali earths. The latter have been shown to leave the body in far greater quantity by the bowel than in the urine."

<sup>1</sup> Studies I-IV were published in the *American Journal of Physiology*.

<sup>2</sup> Meltzer and Auer, *American Journal of Physiology*, 1905, xiv, 386.

<sup>3</sup> MacCallum, "On the Mechanism of the Physiological Action of the Cathartics," Berkeley, 1906, p. 45.

<sup>4</sup> Mendel and Closson, *American Jour. of Physiol.*, 1906, xvi, 45.

Of the alkali earths Mendel himself studied the elimination of strontium<sup>5</sup> and barium salts.<sup>6</sup> For the paths of elimination of calcium salts Mendel refers to Rey.<sup>7</sup> For the salts of these three alkali earths it was variously established experimentally, as stated above, that no matter by what path introduced, they leave the body in far greater quantity by the bowel than in the urine. Magnesium like calcium is an alkali earth and from the rule laid down by Mendel for this class of inorganic compounds it seems to follow that the salts of magnesium also leave the body in far greater quantity by the bowel than in the urine. Are there any experimental facts for such an assumption?

Mendel who does not discuss specially the paths of elimination of magnesium, gives only one reference; it is to Lusk.<sup>8</sup> But here we meet with a statement which is contrary to that assumption. Lusk says that "in carnivorous urine the major part of excreted magnesium is found in the urine, the balance being given off through the intestinal wall to the feces." Lusk mentions no authority for his statement. But the literature on metabolism of inorganic salts contains many studies which bear out this view of Lusk. In the extensive studies of F. Müller,<sup>9</sup> for instance, we find a statement which is exactly like the one made by Lusk. He says "dass beim Fleischfresser der Kalk zum weitaus grössten Theil durch den Darmkanal aus dem Organismus ausgeschieden wird, während die Magnesia . . . hauptsächlich durch den Harn verlässt." Other writers speak of the fraction of magnesium salts which leaves the body through the kidney as being between thirty and forty per cent. However this may be, there is a unanimity of opinion amongst all the investigators, at least as far as we can see, on one point which is of especial interest to us, namely that there is a striking difference between calcium and magnesium with reference to the path of elimination,

<sup>5</sup> Mendel and Thatcher, *American Jour. of Physiol.*, 1904, xi, 5.

<sup>6</sup> Mendel and Sicher, *ibid.*, 1906, xvi, 147.

<sup>7</sup> Rey, *Archiv f. exp. Path. u. Pharm.*, 1895, xxxv, 295. See Rüdell, *ibid.*, 1893, xxxiii, 79.

<sup>8</sup> Lusk, "American Text-Book of Physiology," 2d edition, 1900, Vol. i, p. 527.

<sup>9</sup> Müller, *Zeit für Biol.*, 1884, xx, 334. Heiss, *ibid.*, 1876, xii, 156. Bertram, *ibid.*, 1878, xiv, 335. Renvall, *Skandinavisches Arch. für Physiol.*, 1904, xvi, 94.

the former preferring the alimentary canal, while the latter favors the kidney. In the quoted metabolism experiments the salts of magnesium and calcium were administered by the mouth. As far as we know, there are no experiments on the mode of elimination of magnesium salts when they were given subcutaneously or intravenously. Possibly in the latter cases the elimination through the kidney would prove to be still more favored. At any rate the rule laid down for the elimination of calcium, strontium and barium cannot be applied *a priori* to the elimination of magnesium.

We may therefore claim that there is, to say the least, no experimental evidence based on chemical analysis against the assumption that the kidneys are taking an important share in the elimination of magnesium salts. On the other hand, the above quoted observation of Meltzer and Auer "that animals which urinated frequently had a better chance for recovery" may be taken as an indication in favor of such an assumption. The authors, however, had at that time not made a special study of that question; they speak only of their impressions. Besides, the fact that urination and recovery went hand in hand is not safe evidence that the urination contributed to the recovery. Possibly the recovery takes place through the elimination of magnesium into the intestines and urination occurs on account of the removal of magnesium and in accordance with the above mentioned claim of MacCallum that the presence of magnesium in the blood inhibits urinary secretion; the coincidence of recovery and urination would then mean that recovery is the cause and urination the result and not the contrary.

There is, however, another biological method by which the share of the kidneys in the elimination of the magnesium salts can be systematically studied and which is capable of bringing out reliable results. It is the study of the susceptibility of the animal to the toxic effect of magnesium salts after nephrectomy. Such a series of experiments were carried out by us and the object of this paper is the report of the findings of this research.

THE SUSCEPTIBILITY OF THE ANIMAL TO THE TOXIC EFFECT OF  
MAGNESIUM AFTER NEPHRECTOMY.

*Method.*—The experiments of Meltzer and Auer on the anesthetic effect of subcutaneous injection of magnesium salts were made on seven species of animals and with the sulphates as well as with the chlorides. The present experiments were made on rabbits only, and only magnesium sulphate was employed. Under ether anesthesia double nephrectomy was performed and after recovery from the ether anesthesia variable quantities of magnesium sulphate in molecular solution were injected subcutaneously, using approximately the same region for injection in all animals and observing the same conditions. The results of this research will be presented best by quoting a few abbreviated protocols of experiments.

*Fatal Dose.*—In the following experiment one gram of the salt per kilo animal was injected shortly after nephrectomy.

*Experiment 1.*—June 19, 1906. A series of three rabbits was used.

A. White rabbit, 2,480 grams. 3 p. m. Both kidneys exposed by lumbar route, pedicles firmly ligated and the greatest part of each kidney removed.

3.35. Rabbit recovered from ether, appears quiet and normal. Injected magnesium sulphate ( $\frac{M}{1}$  solution), one gram per kilo weight.

4.10. Animal drowsy, chin rests on floor, head to one side, respiration shallow.

4.35. Lying on side, completely relaxed.

4.43. No conjunctival reflex; reacts feebly to probe in nose.

5.05. Respiration very shallow, 36 per minute.

5.15. Respiration stopped, heart still continues to beat. Animal died one hour and forty minutes after the injection.

B. (Nephrectomized control.) Black rabbit, 2,340 grams; appears to be less robust than A.

3.20. Nephrectomized as in A.

4.00. Does not recover from the anesthesia as rapidly as A; trembles (carbolic intoxication?).

4.20. Hops around, rises on hind legs and seems normal.

June 20. Eats cabbage, appears normal.

June 21, 10 a. m. Did not eat at morning feeding, drinks a little water.

12 noon, found dead.

This animal died about 45 hours after nephrectomy.

C. (Magnesium control.) White and black rabbit, 1,625 grams, not operated.

3.30. Injected magnesium sulphate ( $\frac{M}{1}$  solution) one gram per kilo weight.

At no time was there the slightest sign of a magnesium effect, the rabbit appearing at all times as normal as before the injection.

In this series one gram of magnesium sulphate per kilo animal weight killed the nephrectomized animal one hour and forty minutes after the injection (two hours and fifteen minutes after the operation), whereas the same dose had not the slightest effect upon the normal rabbit and the nephrectomized control animal which received no magnesium, lived forty-three hours and appeared to be normal until a few hours before its death. From the experiments of Meltzer and Auer it appears that a dose less than 1.75 grams per kilo, when given subcutaneously and without massage is not fatal to the rabbit. For the nephrectomized rabbits a dose of only one gram of the salts per kilo animal proved to be fatal; that means an increase of the susceptibility of the animal to the poisonous effect of the magnesium salt nearly equal to fifty per cent.

*Experiment 2.*—Series of two rabbits. June 25, 1906.

A. Gray rabbit, 2,500 grams.

2.15 p. m. Double nephrectomy.

4.10. Injected one gram magnesium sulphate per kilo animal.

4.30. Animal shows signs of beginning anesthesia and paralysis. All symptoms gradually increased and the animal died at 7 p. m. about three hours after the injection.

B. Control (Rabbit C from former experiment). Not operated.

June 25, 4.10 p. m. Injected 1.6 magnesium sulphate per kilo animal.

5.30. Animal greatly relaxed, lid reflex much retarded. Anesthesia and paralysis were at no time complete; the animal soon began to show recovery, and next morning appeared to be perfectly well again.

In this experiment again in the nephrectomized rabbit one gram per kilo killed the animal in a few hours after the injection, while in the normal animal 1.6 grams per kilo was even insufficient to produce complete temporary anesthesia.

In these and in other similar experiments it was then found that one gram per kilo, injected two or three hours after nephrectomy, proved to be a fatal dose, the animal becoming first anesthetized and paralyzed a few hours after the injection.

*Effect of Late Injection.*—The result was different, however, when the injection was not administered until about eighteen hours or more after the nephrectomy. The following protocols will illustrate.

*Experiment 3.*—June 26, 1906. Rabbit A, weighing 1,992 grams, was nephrectomized at 6.30 p. m. Rabbit B, weighing 1,957 grams, was nephrectomized at 6.50 p. m.

A. June 27, 12.35 p. m. (eighteen hours after nephrectomy). Injected magnesium sulphate one gram per kilo in  $\frac{M}{I}$  solution; slight massage.

2. Chin rests on floor, animal remains lying on side when turned over; recovers posture when tail is pressed.

4.45. Animal lying stretched out, relaxed, conjunctival reflex slight. Reacts to probe in nose and on pressing tail.

June 28, 11.40 a. m. Animal shows some recovery, occasionally makes an effort to rise; lid reflex retarded, but definitely present; on pressing the tail, the animal moves head and feet and cries feebly.

Animal died at 1.30 p. m., twenty-five hours after injection, forty-three hours after operation.

B. June 27, 12.40 p. m. About eighteen hours after nephrectomy injected magnesium sulphate one gram per kilo in  $\frac{M}{I}$  solution; massage, stronger than in A.

1.20. Strongly influenced by the salt. Lies flat, head on floor. Raises head when tail is pinched, lid reflex still present.

4.40. Lid reflex completely gone; no reaction to pressure of tail or to probe in nose.

Animal died at 8 p. m., about seven hours after the injection, twenty-five after nephrectomy.

Both animals were injected about eighteen hours after nephrectomy and in both the injected places were massaged, in B a good deal more strongly than in A. Animal A was never completely under anesthesia, had shown later some slight recovery and lived about twenty-five hours after the injection. Animal B was completely under anesthesia and died without recovery about seven hours after the injection.

This last rabbit (B of Experiment 3) was the only animal which lived so short a time as seven hours after the injection when the latter was given late after nephrectomy. Some animals lived twenty-four hours and more after the injection, and one rabbit lived as long as sixty-three hours after an injection of one gram of the salts per kilo animal, which was given twenty-four hours after the double nephrectomy. The narcotizing effect was in this case fairly outspoken and the recovery was very slight, the animal remaining for about three days in the semi-paralyzed and semi-stuporous state.

In general it may be stated that the later after the nephrectomy one gram salt per kilo was given, the less fatal it proved to be and also, perhaps, the less complete was the state of depression; the recovery, however, was in all cases but slight.

*Anesthetic Dose.*—In eight experiments the dose of the salt injected into the rabbits amounted only to 0.8 gram per kilo animal. We shall illustrate the results by one abbreviated protocol.

*Experiment 4.*—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy.

1.50. Injected magnesium sulphate 0.6 grams per kilo in  $\frac{M}{I}$  solution.

3.50. Lying flat, completely relaxed, respiration very slow. Conjunctival reflex present, but diminished.

4.45. Lying completely relaxed on side, conjunctival reflex gone.

6. No change.

June 22, 1.30 p. m. Improved; able to sit up; conjunctival reflex returned.

June 23, 12 noon. Rabbit died, about forty-six hours after injection.

In this experiment with 0.8 grams per kilo, injected very soon after nephrectomy, the animal was completely anesthetized, the depression lasting about twelve hours followed by a moderate degree of recovery. The animal survived the injection by forty-six hours, death apparently being due solely to the nephrectomy. In other experiments with the same dose and under the same conditions the profound anesthesia lasted sometimes more than twenty



FIG. 1. All four rabbits were nephrectomized about twenty-two hours before the photograph was taken. The two animals lying on the table received two hours after the nephrectomy subcutaneous injections of magnesium sulphate, 0.8 per kilo, and about two hours later were completely under the toxic influence of that salt. They remained for about eighteen hours profoundly anesthetized and paralyzed. At the time when the photograph was taken the rabbit on the right hand side showed a slight recovery. The other two rabbits are (nephrectomized) controls.

hours; in other cases again the anesthesia was sometimes a little less complete and the recovery a trifle better than in the last quoted experiment (see Figure). But in the entire series there was no case without distinct anesthetizing and paralyzing effect and none with a fatal outcome due to the magnesium salts. It is hardly necessary to add that in normal rabbits a dose of 0.8 grams per kilo had not the slightest effect.

In a few experiments a dose of 0.8 grams of the salt per kilo animal was given twenty-four hours after nephrectomy. The following protocol is an illustration.

*Experiment 5.*—July 2, 1906. Grey rabbit, 1,810 grams, 11.40 a. m. Double nephrectomy, perfect recovery.

July 3, 1.54 p. m., about twenty-six hours after nephrectomy, injected magnesium sulphate 0.8 grams per kilo in  $\frac{M}{I}$  solution.

3.00 p. m. Animal relaxed, lid reflex very slight.

4.00. Lid reflex gone; no spontaneous movements and no reaction to pressing tail, etc.

10.00. Condition unchanged.

July 4, 10 a. m. Slightly improved, moves head when probe is put in nose, but no reaction to pinching of tail and no lid reflex.

July 5, 10 a. m. Considerably improved; can almost sit up; lid reflex retarded, but present; moves away on pressing tail.

3 p. m. Recovery still more pronounced. Head erect; movements irregular and jerky.

July 6, 8.30 a. m. Found in a dyspnoic state; the animal died five minutes later, about sixty-seven hours after the injection and about ninety-three hours after the operation.

This animal, although injected twenty-six hours after the nephrectomy, became influenced by the salt very rapidly; the depression was very profound and lasted over twenty-four hours. It survived the injection; however, longer than any other animal and the final recovery was more advanced.

Here again, as in the experiments in which a dose of 0.8 grams of the salt was given soon after the nephrectomy, the extent of the effect of the injection was somewhat variable, but the effect was on the one hand never missing and on the other hand never fatal.

In general it may be said that for a dose of 0.8 gram per kilo of the salt there was not a marked difference between the effects of the injections whether given soon after nephrectomy or twenty-four hours later, and this difference certainly cannot be compared



with the striking differences observed in the experiments with one gram per kilo animal.

*Minimum Dose.*—We have made seven experiments in which a dose of only 0.6 gram of the magnesium salt per kilo rabbit was injected. The following few abbreviated protocols will illustrate the corresponding results.

*Experiment 6.*—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy. Completely recovered.

3 20. Injected 0.6 gram of magnesium sulphate per kilo animal in  $\frac{M}{I}$  solution.

4.45 and 6 p. m. Appears normal in every way. Animal remained normal until its death which occurred June 24, 8 p. m., about seventy-two hours after injection and seventy-four hours after operation.

In this animal 0.6 gram per kilo had apparently no effect whatsoever.

*Experiment 7.*—July 6. White rabbit, 1,610 grams.

11 a. m. Double nephrectomy; complete recovery.

2 p. m. Injected 0.6 gram magnesium sulphate per kilo in  $\frac{M}{I}$  solution.

3.15. Some loss of muscular control, especially of hind legs; reflex much retarded.

5.15. Stretched out, completely relaxed; lid reflex sluggish but present; reacts promptly to pinch of tail.

8.15. Shows some recovery; able to sit up.

July 7, 9.30 a. m. No further recovery. General muscular weakness, especially of the anterior part, reacts sluggishly to stimulation, lid reflex retarded. Animal remained in the same state until it died July 8, 7 p. m. It lived fifty-three hours after the injection and fifty-six hours after the operation.

This animal was moderately but distinctly influenced by a dose of 0.6 gram per kilo. It recovered from a major part of the depressing influence within five hours after the injection, but retained some degree of depression until death which was apparently due solely to the nephrectomy.

These two experiments represent both extremes of the results. Out of seven experiments with 0.6 per kilo three have shown practically no signs of depression. In the other four animals anesthesia and paralysis were present in a variable degree, but were never complete and lasted only a few hours. In two the recovery was not complete.

In two experiments the injection of 0.6 gram per kilo was given about twenty-four hours after the nephrectomy. In one the de-

pression was hardly noticeable and in the other the depression was well marked, but of only short duration with complete recovery.

Apparently in nephrectomized rabbits a dose of 0.6 gram per kilo is bordering on the minimum toxic dose and the various uncontrollable minor factors which frequently influence to a slight extent the degree of absorption cause this dose to be sometimes without any effect and at other times to produce a well defined, though only temporary influence.

*Cumulative Effect.*—An interesting point is the question of the cumulative effect. In normal animals the effect of several sub-minimum doses administered at various times is not equal to the effect of the sum of these doses when given in single injection on account of the elimination which takes place during the intervals between the injections. It was different, however, with the behavior of magnesium salts in nephrectomized animals, as can be seen in the following experiment.

*Experiment 8.*—July 6, 1906. White rabbit, 1,740 grams.

12 noon. Double nephrectomy.

2.15 p. m. Injected magnesium sulphate 0.3 gram per kilo in  $\frac{M}{1}$  solution.

3.30 and 4 p. m. Animal normal.

4.15. Injected again, 0.3 gram per kilo of the magnesium salt.

4.30. Animal lying on side, completely under anesthesia; lid reflex gone.

8.15. Seems somewhat improved; moves head.

8.20. Injected again 0.3 gram per kilo.

11 p. m. Animal profoundly under anesthesia.

July 7. 9 a. m. Somewhat improved; moves head and feet, but lid reflex still absent.

9.25. Injected again (fourth time) 0.3 gram per kilo.

12 noon. Profoundly under anesthesia; respiration very shallow.

Animal died at 2 p. m.

In this animal two subminimum doses of 0.3 gram per kilo given two hours apart had after the second dose at least as much effect as 0.6 gram per kilo given in one dose. A similar result was obtained when a dose of 0.2 gram per kilo was given hourly. About half an hour after the third dose the animal became fairly well anesthetized and recovered again after a few hours. In these cases, if the interval between the injections did not exceed two or three hours, the cumulative effect was perfect and in fact the sum of two or more doses seemed to be even more effective than a cor-

responding single dose. Possibly the cumulative effect might have been due to the fact that a single dose was given in only one place, while the several smaller doses were given in several different places, the latter circumstance favoring absorption.<sup>10</sup>

*Discussion.*—The following facts were brought out by the foregoing series of experiments.

A dose of one gram magnesium sulphate per kilo animal, when injected subcutaneously within two or three hours after double nephrectomy proved invariably to cause death a few hours after injection. In normal animals such a constant result could be obtained only with a dose of 2 grams per kilo.

A dose of 0.8 gram per kilo, injected within two or three hours after nephrectomy, was never fatal to the animal, but the animal became invariably more or less completely anesthetized and paralyzed. In normal animals such a constant effect could be attained only with a dose not less than 1.6 gram per kilo.

The susceptibility to the anesthetic and toxic effects of the magnesium salts was therefore in the nephrectomized animals increased with about fifty per cent. This increase of susceptibility is apparently due to the decrease in the facility of elimination of the salts by the urine. These experiments therefore tend to show that normally the kidneys carry off at least fifty per cent. of the injected salts. We should, however, lay little stress upon the exact figures. But we are, we believe, justified in stating that the experiments demonstrate conclusively *that the kidneys play an essential part in the elimination of the magnesium salts.*

In harmony with this conclusion is the observation which was made upon the cumulative action of the magnesium salt in the nephrectomised animals. The effect of the two or three injections of subminimum doses was at least equal to the sum of these quantities given in a single dose; a fact which can only be explained by the assumption that during the intervals none of the salt was eliminated. This observation would seem to contain the suggestion that at least during the first few hours after an injection no elimination takes place except through the kidneys. We shall, however, not dwell too much on this side of the question.

<sup>10</sup> Meltzer, *Jour. of Exper. Med.*, 1901, v, 643.

A further instructive fact is the observation that the profound anesthesia and paralysis produced by an injection of a dose of 0.8 gram per kilo lasted undiminished twelve to twenty hours and sometimes a good deal longer. Furthermore the recovery which finally took place was only moderate, the animal remaining until death in a pronounced state of paresis and stupor in sharp contrast with the nephrectomized control animal. In normal animals the anesthesia which was brought on by an efficient, but not fatal dose lasted at the utmost two hours and then the recovery was complete. This fact again can be best explained by the assumption that in the nephrectomized animal no fraction of the absorbed salts is eliminated for twelve or eighteen hours.

An interesting fact finally is the observation that a dose of one gram per kilo which is invariably fatal when given soon after nephrectomy is no longer fatal when injected eighteen to twenty-four hours after nephrectomy, although it still produces deep anesthesia; in other words eighteen to twenty-four hours after nephrectomy a dose of one gram per kilo acts like a dose of 0.8 per kilo injected soon after nephrectomy. This seems to indicate that some time after nephrectomy vicarious paths of elimination develop which thus assist in converting a fatal dose into only an anesthetic dose. This assumption would also explain the cause of the recovery from deep anesthesia twelve or eighteen hours after an injection of a dose of 0.8 gram per kilo; as at that time some of the salt becomes eliminated.

The degree of this vicarious elimination, however, is apparently very small; it never converted a fatal dose or an anesthetic dose into a harmless one, and the recovery from the anesthesia is only moderate, the animal generally remaining until death under a considerable influence of the salts.

The results which were obtained by us in the study of the effect of nephrectomy upon the toxicity of magnesium salts are in marked contrast to the results obtained by Meltzer and Salant<sup>11</sup> in their study of the effect of nephrectomy upon the toxicity of strychnin. Strychnin is generally assumed to be eliminated essentially through the kidney. Meltzer and Salant nevertheless found that the mini-

<sup>11</sup> Meltzer and Salant, *Journ. of Exper. Med.*, 1905, vi, 107.

imum toxic dose is for nephrectomized rabbits the same as for normal ones. For magnesium we found that for nephrectomized rabbits the dose is half of that which is toxic for the normal animals. Furthermore, the cumulative effect of strychnin is remarkably small. In intervals of two or three hours subminimum doses can be given, until they equal the sum of two or three times the toxic dose before any toxic symptoms will appear. For magnesium we found that the toxic effect will appear as soon as the sum of the subminimum doses becomes equal to the single minimum dose which is capable of producing an effect. Finally in nephrectomized rabbits no dose of strychnin was ever observed to produce continuous convulsions for any length of time; the animals either succumb soon or the convulsions gradually subside. With magnesium we observed that in nephrectomized rabbits the anesthesia may last uniformly for twenty-four hours and longer. Strychnin apparently finds soon after nephrectomy a satisfactory vicarious path for its elimination from the body, at least in rabbits. It should be mentioned that for guinea pigs Meltzer and Langmann<sup>12</sup> observed that within the first three hours after nephrectomy the toxic dose is indeed smaller than for the normal animal and that at that period in some animals a subminimum dose produced a continuous vibration which the authors termed a *subtetanic reaction*. They explained these observations by the assumption that in guinea pigs during the first few hours after nephrectomy vicarious paths for the elimination of strychnin are not yet developed.

Vicarious paths for elimination of magnesium even in rabbits do not develop until late after the nephrectomy and even then only in an unsatisfactory manner.

*Conclusions.*—Magnesium salts when introduced subcutaneously are eliminated to a great extent through the kidneys. In nephrectomized rabbits the susceptibility to the toxic effect of magnesium salts is increased by about fifty per cent.

The profound anesthesia which a toxic dose of magnesium produces in nephrectomized rabbits may be continuous for twenty-four hours and longer.

The cumulative effect of magnesium salts in nephrectomized

<sup>12</sup> Meltzer and Langmann, *Journ. of Med. Research*, 1903, ix, 19.

rabbits is very striking. The effect of several subminimum doses is equal to the effect produced by the sum of these doses given in a single injection.

A dose which when given soon after the nephrectomy is fatal, causes only a non-fatal anesthesia when given eighteen hours or later after the nephrectomy. Probably at that period vicarious paths develop sufficient for elimination of a fraction of the salts.

It is, probably for the last mentioned reason, that the profound anesthesia produced by a proper dose of the magnesium salts is partially recovered from about twelve to eighteen hours after nephrectomy.

## Über die diuretische Wirkung des Thymins.

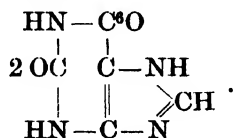
Von

P. A. Levene.

(Aus dem Rockefeller Institute for Medical Research, N. Y.)

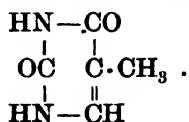
(Eingegangen am 1. Mai 1907.)

Die Erfahrung über die diuretische Wirkung der Purinbasen hat zulichte gebracht, daß die Wirkung hauptsächlich auf zwei Bedingungen beruht: Die Anwesenheit im Molekül von zwei Oxygruppen und die Substitution einer oder mehrerer Wasserstoffe durch die Methylgruppe. Die wirksamsten Substanzen sind die methylsubstituierten Xanthine. Nun ist von Fischer erklärt worden, daß Xanthine das 2,6-Di-oxypurin ist mit der graphischen Formel:



Im Theophyllin sind die Methylgruppen in der Stellung 1:3, im Theobrominen in Stellung 3—7, im Coffein in 1,3,7 substituiert. Es ist daraus ersichtlich, daß die beiden Oxygruppen und zum mindesten eine Methylgruppe im Pyrimidinringe gelagert sein müssen. Diese Betrachtung führt natürlicherweise auf den Gedanken, daß auch Pyrimidinbasen, welche den erwähnten Purinen eine analoge Zusammensetzung geben, in ihrer physiologischen Wirkung den Purinen ähnlich sein würden.

Diese theoretischen Betrachtungen wurden bestätigt durch die Erfahrung über die physiologische Wirkung des Thymins. Diese Base ist eine 5-Methyl-2-6-di-oxypyrimidin



Im Laufe einer Arbeit, die Dr. Sweet und ich<sup>1)</sup> an einem Hunde mit Ecks Fistula ausführte, wurde es bemerkt, daß nach Thyminfütterung eine stark ausgesprochene Diurese folgte. In jenem Experiment wurde das Tier eine Zeitlang auf purinfreie Kost erhalten und der Urin in dreitägigen Perioden durch Katheterisieren genau gewonnen. Zwei ausgeführte Versuche waren in ihren Resultaten ganz übereinstimmend. Nun schien es wünschenswert, die Zahl der Beobachtungen zu vergrößern. Durch die Freundlichkeit des Herrn Dr. Carrel stand uns ein anderes Tier mit Ecks Fistula zur Verfügung.

Nun war das Tier aber so klein, daß Katheterisieren nicht gut ausgeführt werden konnte, und so wurde der Harn in einem Stoffwechsellkäfig gesammelt. Das Futter bestand aus Plasmon 12,0, Crackerdust 60,0, Specie 10,0, welches in 125 ccm Wasser gemischt wurde. Außerdem bekam das Tier dreimal täglich genau gemessene Quantität Wasser. Die Operation fand statt am 15. Dezember, die Beobachtung begann am 11 März.

Aus der folgenden Tabelle ist es klar, daß auch bei diesem Versuche eine starke Diurese erfolgte. Es ist bemerkenswert, daß nach der zweiten Fütterung die Harnausscheidung sich auf eine längere Zeit erstreckte.

Auch dieses Experiment wurde an einem Hund mit Ecks Fistula ausgeführt, weil unter diesen Bedingungen das Thymin nicht die schnelle Zersetzung erfuhr, welche Steudel bei normalen Hunden beobachtete.

Wir hoffen, die Beobachtungen an einer größeren Anzahl von Tieren und mit einer größeren Anzahl von Pyridinbasen zu erweitern.

Tabelle I.

April 27 — 28 — 29	1500
30 — Mai 1 — 2	2020
3 — 4 — 5	1700
6 — 7 — 8	1000
16 — 17 — 18 (L GR. Adenin sulfat.)	2190
19 — 20 — 21	3000
22 — 23 — 24 (10,0 Nuclein Acid.)	3420

<sup>1)</sup> The Journ. of Exper. Med. 9, 229, 1907.



April 25 — 26 — 27	2710
28 — 29 — 30	2560
30 — Juni 1 — 2 (3,0 Thymin)	3005
3 — 4 — 5	3020
6 — 7 — 8 (6,0 Thymin)	4425
9 — 10 — 11	2800
12 — 13 — 14	2720
15 — 16 — 17	2800
18 — 19 — 20	2650

Tabelle II.

Datum	Wasser	Urin
11. März	450 ccm	240 ccm
12. „	80 „	100 „
13. „	200 „	150 „
14. „	100 „	75 „
15. „	100 „	100 „
16. „	250 „	150 „
17. „		
18. „	150 „	175 „
19. „	75 „	50 „
20. „	50 „	50 „
21. „	90 „	40 „
22. „	75 „	120 „
23. „	150 „	125 „
24. „		
25. „	225 „	200 „
26. „	300 „	220 „
27. „	75 „	125 „
28. „	200 „	75 „
29. „	140 „	170 „
30. „		
31. „	450 „	350 „
1. April		
2. „	250 „	150 „
3. „	100 „	150 „
4. „	200 „	100 „
5. „ <sup>1)</sup>	250 „	150 „

<sup>1)</sup> 2,0 g Thymin.

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6. April	500 ccm	350 ccm
7. „	270 „	150 „
8. „ <sup>1)</sup>	125 „	160 „
9. „	200 „	325 „
10. „	200 „	150 „

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<sup>1)</sup> 2,0 g Thymin.

## Über die Tryptophangruppe im Proteinmolekül.

Von

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(Aus dem Rockefeller Institute for Medical Research, N. Y.)

(Eingegangen am 1. Mai 1907.)

Die in dieser Mitteilung berichteten Resultate sind im Laufe einer Untersuchung über den quantitativen Gehalt verschiedener Proteine an Tryptophan erhalten worden. Ein Teil der Befunde ist in die Redaktion des „Journal of Biological Chemistry“<sup>1)</sup> den 29. November vergangenen Jahres eingegangen, ein anderer Teil wurde in der Sitzung der Physiologischen Gesellschaft am 30. Dezember 1906 vorgetragen und deswegen erlauben wir uns, trotz der unterdessen erschienenen sehr schönen Arbeit von Neuberg und Popowsky<sup>2)</sup>: „Über die Halogenderivate des Tryptophans“ diese Untersuchung zu veröffentlichen.

Um die Ausführung der ursprünglichen Aufgabe zu ermöglichen, wurde es nötig: 1. ein bequemes Verfahren zur Tryptophanbestimmung auszuarbeiten und 2. die Bedingungen für die Maximalausbeute an der Substanz aufzufinden. Es erwies sich möglich, Tryptophanbestimmung durch Titrieren mittels Bromwasser auszuführen. Als Endreaktion wurde der Übergang des violetten Körpers in das Gelbe gebraucht. Die genauen Aufgaben sind in dem „Journal of Biological Chemistry“ angegeben.

Die Spaltung der Proteine sollte nicht durch proteolytisches Ferment, sondern durch Barytwasser ausgeführt werden. Nun stieß man bald auf eine unerwartete Schwierigkeit. Bei der Arbeit mit reinem Tryptophan oder mit der Fraktion, die durch Mercurisulfat nach Hopkins und Cole aus dem mit Trypsin verdauten Casein erhalten wird, erscheint die violette Farbe des

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1) Journ. of Biol. Chem. 2, 481, 1907.

2) Biochem. Zeitschr. 2, 357, 1907

Essigätherauszuges sofort nach Zugabe der ersten Tropfen von Bromwasser. Bei der Barytspaltung war dies der Fall, nur wenn die Spaltung tiefgreifend war, sonst trat die Violettfarbe nur nach gehöriger Zugabe vom Reagens ein, und dabei war die Farbe nicht rein violett, sondern mit einem Stich ins Braune. Diese Unregelmäßigkeit schien uns durch die Anwesenheit von Peptiden verursacht zu sein, und um diese zu entfernen, wurde die Mercurisulfatfraktion mit Phosphorwolframsäure behandelt, mit der Absicht, das Tryptophan im Filtrate zu finden.

Es erwies sich aber, daß bei milder Spaltung das Filtrat vom Phosphorwolframsäureniederschlag gar keine Tryptophanreaktion gebende Substanzen erhielt, während diese im Niederschlag vorhanden waren. Beim Steigern des Grades der Hydrolyse nahm die Intensität der Bromfärbung im Phosphorwolframsäureniederschlag ab und im selben Maße im Filtrate zu, bis endlich alle Tryptophanreaktion gebende Substanz im Filtrate erschien. Diese Beobachtung veranlaßte eine genauere Untersuchung der zwei mit Brom reagierenden Fraktionen.

Aus dem Filtrate ließ sich leicht Tryptophan in üblichen perlmutterglänzenden Kristallen darstellen. Ein ganz reines Präparat erhält man durch Umkristallisieren aus 50% Essigsäure.

Die Analyse eines solchen Präparates hatte die folgende Zusammensetzung:

Gefunden		Berechnet für
		$C_{11}H_{12}N_2O_2$
C	63,84%	64,64%
H	5,31%	5,93%

Alle Versuche, auch aus dem Phosphorwolframsäureniederschlag die Substanz zu kristallisieren, waren vergeblich. Man mußte deswegen denken, daß entweder in dieser Fraktion das Tryptophan mit solchen Substanzen verunreinigt war, die die Kristallisation verhinderten, oder daß sie eine kompliziertere Substanz, die mit Brom dem Tryptophan ähnlich reagierte, enthielt. Von Phosphorwolframsäure befreit, besaß die Fraktion die folgenden Eigenschaften: Sie reagierte ganz schwach alkalisch, gab eine sehr schwache, aber ganz unzweifelhafte Biuretkreaktion, sowie die Xantoprotein- und auch die Adamkiewitz-Reaktionen, gab absolut keine Millonreaktion und enthielt keinen Schwefel.

Mit Silbernitrat wurde es möglich, eine Fraktion zu erhalten, die mit Brom dem Tryptophan ähnlich reagierte, und die scheinbar die Biuretreaktion nicht mehr gab. Leider reichte die Menge des Materials zur genaueren Untersuchung nicht aus. Mit Silbernitrat und vorsichtigem Neutralisieren mit Ammoniak läßt sich eine andere Fraktion erhalten, die sich aber vom ursprünglichen Materiale nicht wesentlich unterschied. Es war nun möglich zu denken, daß in der Phosphorwolframsäurefraktion ein Tryptophanpeptid vorlag, und ferner, da die Fraktion auf blauer Lackmus gar nicht reagierte, und da bei tryptischer Verdauung der Gelatine gerade ein Anhydridpeptid vorkommt, wurde die Fraktion auf die Anwesenheit von Substanzen solcher Natur untersucht, nämlich es wurde das Verhalten dieser Fraktion zum frisch gefällten Kupferoxyd untersucht. Man dürfte nämlich erwarten, daß das Tryptophan mit diesem Reagens ein Salz zu bilden fähig ist, während dem Anhydride die Fähigkeit fehlen soll. Nun erwies sich, daß Tryptophan, mit Kupferoxyd erhitzt, ein unlösliches, wahrscheinlich basisches Salz bildet, so daß das Filtrat von diesem Salze mit Brom gar nicht reagierte, im Gegenteil bildete die Phosphorwolframsäurefraktion eine wasserlösliche Kupferverbindung, die alle bromreagierende Substanz enthielt. Es ließ sich nun an zwei Möglichkeiten denken: eine, daß die Phosphorwolframsäurefraktion kein freies Tryptophan enthielt, und die zweite, daß sie eine Substanz enthielt, die die Bildung des basischen Salzes verhinderte. Um die Sache weiter zu erforschen, wurde es versucht, eine Lösung der Phosphorwolframsäurefraktion, in welcher eine bekannte Menge Tryptophan aufgelöst war, mit Kupferoxyd zu behandeln. Dabei erwies sich, daß nur etwa zwei Drittel des aufgelösten Tryptophans in das unlösliche Salz übergeführt war, das andere bildete ein lösliches Salz.

Da alle anderen Verfahren, die Substanz aus der Phosphorwolframsäurefraktion zu gewinnen, versagten, so wurde beschlossen, die Bromverbindungen darzustellen und sie mit denen des Tryptophans zu vergleichen. Als diese Versuche im Gange waren, erschien die schöne Arbeit von Neuberg und Popowsky, welche uns unsere Aufgabe viel erleichterte.

Nun wurde die Bromierung durch Bromwasser ausgeführt und die Bromkörper mit Essigäther extrahiert. Das letzte Ver-

fahren wurde gewählt, weil Brom auch peptonartige Körper fällt, und man dürfte glauben, daß diese vom Essigäther nicht aufgenommen würden. Die Essigätherauszüge wurden im Vakuum-exsiccator verdunstet und in einigen Fällen im Toluolbad, in anderen im Exsiccator bis zum konstanten Gewicht getrocknet. In späteren Versuchen wurden die Bromniederschläge zentrifugiert, auf der Zentrifuge mit Wasser gewaschen, aus den Röhren mittels Alkohol in Schalen übertragen und dann getrocknet.

Es wurde beabsichtigt, die Zusammensetzungen der violetten Körper mit denen, die sich beim Übergange dieses in das Gelbe bilden, zu vergleichen. In einem Vorversuche wurde gewöhnlich die Menge des Bromwassers, welche zum Überführen des sich gebildeten violetten Körpers in das Gelbe notwendig war, bestimmt, und dann wurde zur Hauptlösung eine Menge Bromwasser, etwas kleiner als die berechnete, zugefügt. Beim Behandeln mit Bromwasser einer Tryptophanlösung bildete sich bald ein violetter Niederschlag, bei weiterem Behandeln entsteht ein gelber Niederschlag, welcher bei stärkerem Schütteln wieder in das Violette übergeht, und endlich bildet sich der bleibend gelbe Körper. Beim Bromieren der Phosphorwolframsäurefraktion entsteht sofort der gelbe Niederschlag, der beim Schütteln anfangs in das Violette übergeht.

Die Analyse einiger auf der angegebenen Weise dargestellten Präparate ergaben die folgenden Zahlen:

Präparat I: Aus der Phosphorwolframsäurefraktion dargestellt.

Bromwasser nicht ganz bis zum Verschwinden des violetten Körpers; extrahiert mit Essigester.

0,1242 g Substanz gaben AgBr 0,1051 g; Br = 36,01%.

Präparat II: Auf dieselbe Weise dargestellt.

0,1320 g Substanz gaben AgBr 0,1093 g; Br = 35,24%.

Präparat III: Die Phosphorwolframsäurefraktion mit Silbernitrat gefällt, der Niederschlag von Silber befreit und die Bromverbindung wie in den anderen Versuchen gewonnen.

0,1174 g Substanz gaben AgBr 0,0807 g; Br = 29,25%.

Präparat IV: Aus dem Filtrat von der obigen Silberfällung durch Behandeln mit Silbernitrat und Ammoniak erhalten. Bromierung wie sonst ausgeführt.

0,1252 g Substanz gaben AgBr 0,1043 g; Br = 35,45%.

0,2180 g Substanz gaben 15,5 ccm Stickstoff (über 50% KOH) bei  $T^{\circ} = 27$  und  $P = 763,8$  mm;  $N = 8,13\%$ .

Präparat V: Durch Behandeln der Phosphorwolframsäurefraktion mit Überschuß von Bromwasser erhalten. Der Niederschlag mit Wasser auf der Zentrifuge gewaschen und im Vakuum bis zum konstanten Gewicht getrocknet.

0,2162 g Substanz gaben bei Verbrennung 0,2729 g  $\text{CO}_2$  und 0,0725 g  $\text{H}_2\text{O}$ ;  $C = 34,42\%$ ;  $H = 3,75\%$ .

0,2502 g Substanz gaben bei Verbrennung 0,3178 g  $\text{CO}_2$  und 0,0794 g  $\text{H}_2\text{O}$ ;  $C = 34,63\%$ ;  $H = 3,55\%$ .

0,1039 g Substanz gaben  $\text{AgBr}$  0,4338 g;  $\text{Br} = 43,38\%$  für  $\text{C}_{11}\text{H}_{10}\text{N}_2\text{O}_2\text{Br}_2$ .

	Berechnet	Gefunden	
C	36,45%	34,42%	34,63%
H	2,78%	3,75%	3,55%
Br	44,14%	43,38%	

Zum Vergleich mit diesen Körpern wurden die Bromprodukte des Tryptophans dargestellt.

Präparat I: Bromierung ausgeführt mit Bromwasser und das Produkt mit Essigester ausgezogen. Bromwasser nicht bis zum Verschwinden der violetten Farbe zugegeben.

0,1537 g Substanz gaben  $\text{AgBr}$  0,1494 g;  $\text{Br} = 41,39\%$ .

Präparat II: Auf ähnliche Weise dargestellt.

0,1237 g Substanz gaben  $\text{AgBr}$  0,1174 g;  $\text{Br} = 40,39\%$ .

Präparat III. Auf ähnliche Weise dargestellt.

0,1328 g Substanz gaben  $\text{AgBr}$  0,1316 g;  $\text{Br} = 42,17\%$ .

Präparat IV: Aus wässriger Lösung durch Fallen mit Bromwasser. Nur die Hälfte der berechneten Menge Brom zugegeben.

0,1218 g Substanz gaben  $\text{AgBr}$  0,0829 g;  $\text{Br} = 28,96\%$ .

Für  $\text{C}_{11}\text{H}_{11}\text{N}_2\text{O}_2\text{Br}$ :  $\text{Br} = 28,71\%$ .

Präparat V: Wie Präparat IV dargestellt, nur wurde Bromwasser in einer Menge, die zum Verschwindenbringen der violetten Farbe nicht ganz ausreichte, zugefügt. Der Niederschlag auf der Zentrifuge mit Wasser gewaschen und getrocknet.

0,1426 g Substanz gaben 0,1393 g  $\text{AgBr}$ ;  $\text{Br} = 41,57\%$ .

Präparat VI: Wie oben dargestellt. Bromwasser im Überschuß zugefügt.

0,1142 g Substanz gaben AgBr 0,1207 g; Br = 44,98%.

Für  $C_{11}H_{10}N_2O_2Br_2$  berechnet Br = 44,14%.

Alle untersuchten Körper enthielten das Br scheinbar in substituierter Form. Sie besaßen keine Eigenschaften der Hydrobromide oder der Perbromide. Es gelang nicht, das Brom mittels Silbersalzen zu entfernen, und auch die gelben Präparate mit Maximalbromgehalt besaßen nicht die Fähigkeit, Jod aus Jodkaliumlösung freizusetzen, auch gaben sie kein Brom an freies Tryptophan ab. Doch ist es wahrscheinlich, daß auch beim Bromieren mit Bromwasser die gelben Niederschläge, die direkt nach der Bromzugabe sich bilden und beim Schütteln in violette übergehen, Perbromide sind.

Aus unseren Versuchen scheint man berechtigt anzunehmen:

1. daß die violetten Körper, die bei der Zugabe von Bromwasser zu Verdauungsprodukten entstehen, ein Gemisch von Mono- und Dibromid des Tryptophans sind;

2. daß bei der Zugabe eines Überschusses von Bromwasser sich ein Dibromid bildet;

3. daß bei der Spaltung des Proteinmoleküls sich zuerst scheinbar ein komplizierteres Produkt als das Tryptophan bildet.

Mit der Untersuchung über die Beschaffenheit dieses Körpers, wie auch die Konstitution des Dibromids des Tryptophans sind wir jetzt beschäftigt.

Wie erwähnt, ist der Ausgangspunkt dieser Arbeit, die quantitative Bestimmung des Tryptophans in verschiedenen Proteinen, jetzt im Gange.



## Über die Hydrolyse der Proteine mittels verdünnter Schwefelsäure.

Von

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(Aus dem Rockefeller Institute for Medical Research, N. Y.

Von dem Biochemical Department of Harvard University, Boston.)

*(Eingegangen am 1. Mai 1907)*

Die älteren Beobachtungen über die Einwirkung verdünnter Mineralsäuren auf Proteine haben nicht viel zur Kenntnis dieser Körper beigetragen. Die erste eingehende Arbeit auf diesem Gebiete wurde von F. Goldschmidt<sup>1)</sup> ausgeführt. Darauf folgten die Untersuchungen von Lawrow<sup>2)</sup> und seinen Mitarbeitern. Alle diese Arbeiten waren qualitativer Natur. Es handelte sich immer darum, die Eigenschaften der dabei entstehenden Körper zu untersuchen oder den Umfang der Veränderung, die dadurch im Proteinmolekül verursacht war, aufzuklären.

Die vorliegenden Untersuchungen beruhen auf ganz anderen Betrachtungen. Es ist durch die Arbeiten von Fischer und seinen Mitarbeitern schon jetzt bis zu einem gewissen Grade aufgeklärt worden, daß die Struktur der Peptide einen Einfluß auf die Resistenz dieser Körper gegen hydrolysierende Agentien ausübt. Diese Beobachtung macht es nötig, bei dem Studium der Eigenschaften einzelner Proteine oder Gruppen von Proteinen, wie auch Verdauungsprodukte der Proteine (Proteose, Peptone), auch den Widerstand dieser Körper gegen die proteolytische Wirkung von Mineralsäuren oder Fermenten zu untersuchen. Die Untersuchung schien uns dringend zu sein auch wegen einiger biologisch wichtiger Fragen. Es ist nämlich noch nicht aufgeklärt worden, inwieweit die Verdaulichkeit eines Proteins mit ihrem physiologischen Nährwert in Verbindung steht. Wie bekannt,

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<sup>1)</sup> Inaug.-Diss. Straßburg 1898.

<sup>2)</sup> Zeitschr. f. physiol. Chemie 43, 447, 1905.

ist die ziemlich gut verdauliche Gelatine ein unbefriedigendes Nahrungsmittel, während Eialbumen viel schwerer verdaulich ist, aber einen viel besseren Nährwert besitzt. Um diese Punkte aufzuklären, muß man erst die Verdaulichkeit einzelner Proteine klarstellen.

Die Untersuchung hat auch einen anderen Zweck. Seit längerer Zeit ist einer von uns mit der Zusammensetzung der Verdauungsprodukte des Proteins beschäftigt, und nun kam es darauf an, die Bedingungen auszufinden, welche zum Gewinnen einzelner Proteosen oder Peptone am günstigsten sind. In der vorliegenden Mitteilung sollen nur die Ergebnisse der Spaltungsversuche von drei Proteinen berichtet werden: nämlich der Gelatine, des Caseins und des Edestins.

Die Hydrolyse wurde ausgeführt im Autoklav mit Wasser und verdünnter Schwefelsäure von steigender Konzentration, bis endlich das Spaltungsprodukt keine Biuretreaktion mehr zeigte. Die Temperatur des Ölbadesschwankte zwischen 140 und 170° C. Bei der Bestimmung der Stickstoffverteilung wurden die folgenden Fraktionen in Betracht genommen. Das Filtrat, welches nach halb Sättigung mit Zinksulfat, durch Sättigung mit demselben Reagens und in jenem durch Fällen mit 10 prozentiger Phosphorwolframsäure entstehen.

### Gelatine.

In folgender Tabelle sind die Resultate der Analysen angegeben :

Stärke der Säure . . . .	0,0	0,5%	1,0%	1,36%	2,1%	3,1%	5%	8%
Temperatur des Bades .	140—165	140	150—170	150 170	160—170	140 160	140 175	150° C.
Länge der Hydrolyse . . .	4 St.	4 St	3 St	4 St.	4 St	4 St.	4 ½ St	4 St.
Total N in Lösung . . . .	2,673	1,5088	1,4406	1,4927	1,4945	1,5036	1,8476	1,24
N im Filtrat von halbsatur. H <sub>2</sub> O Lösung von ZnSO <sub>4</sub> .	0,6279	1,5563	1,4025	1,5365	—	—	—	—
Ditto, ditto im Prozentsatze des Ganzen . . . . .	23,48%	97,37%	97,3%	—	—	—	—	—
N im Filtrat von vollsatur. H <sub>2</sub> O Lösung von ZnSO <sub>4</sub> .	0,56918	1,0608	1,4206	—	—	—	—	—
Ditto, ditto im Prozentsatze des Ganzen . . . . .	21,29%	67,60%	98,6%	—	—	—	—	—
N im Filtrat von P. W. Acid. Präcipitat . . . . .	0,05684	0,1221	0,2098	0,357	0,7236	0,6597	0,9542	1,0696
Ditto, ditto im Prozentsatze des Ganzen . . . . .	2,12%	7,63%	15,56%	23,9%	49,01%	43,89%	51,65%	85,44%

10,0 g Gelatine wurden in 200 ccm Lösung aufgenommen. Im Experiment mit 1 prozentiger Schwefelsäure war nach dem Abkühlen des Autoklaven das Volumen der Lösung auf 125 ccm vermindert gefunden, bei 2,1% bis auf 80 ccm und im Versuche mit 5% und 8% Säure wurde das Volumen vergrößert gefunden. Beim Versuche mit 3,1% und mit 5% war die Biuretreaktion kaum ausgesprochen und im Experimente mit 8% kam die Reaktion nicht mehr vor.

### Casein.

Ein Präparat von Kahlbaum, dargestellt nach Hammarsten, ist zu dem Experiment gebraucht.

10,0 g Casein und 200 ccm Säure wurden zu jedem Versuche in Anwendung genommen.

Die Verteilung des Stickstoffes wurde in ähnlichen Fraktionen wie bei der Hydrolyse der Gelatine vorgenommen. Die Ergebnisse der Analyse folgen:

Stärke der Säure . . . . .	25% <sup>1)</sup>	0,5% <sup>2)</sup>	3%	5%	8%	10%	15% <sup>3)</sup>	15% <sup>4)</sup>
Total N . . . . .	0,125	1,2400	1,0810	1,3053	1,7338	1,303	1,488	1,418
N im Filtrat von halbsatur. ZnSO <sub>4</sub> Lösung . . . . .	0,0252	0,4235	1,0076	1,3050	1,603	1,264	—	—
% von total N in obiger Lösung . . . . .	15,27%	34,31%	92,7%	99,98%	92,46%	90,07%	—	—
N im Filtrat von satur. ZnSO <sub>4</sub> Lösung . . . . .	0,01358	0,2695	0,91125	1,2545	1,4850	1,265	1,323	—
% von total N in obiger Lösung . . . . .	8,2%	21,74%	84,29%	96,1%	82,76%	90,08%	88,9%	—
N im Filtrat von P. W. Acid. Precipitat . . . . .	0,0087	0,1120	0,3688	0,661	0,609	0,645	0,8125	0,8750
% von total N in obiger Lösung . . . . .	5,3%	9,03%	34,11%	51,48%	31,08%	46,30%	54,6%	61,7%

<sup>1)</sup> Ein Teil des Caseins ist ungelöst geblieben. Die Lösung schien stark kolloidal gewesen zu sein, so daß Filtrieren unmöglich war. Nach weiterer Zugabe von Säure bildete sich ein reichlicher, flockiger Niederschlag; dieser wurde abfiltriert und gewaschen. Im Filtrate wurde die Stickstoffverteilung bestimmt.

<sup>2)</sup> Auch in diesem Experimente ist ein Teil des Caseins ungelöst geblieben.

<sup>3)</sup> Nach Zugabe von Natronlauge bildete sich ein Niederschlag (Phosphorsäure). Die Biuretreaktion noch gut ausgesprochen.

<sup>4)</sup> Die Hydrolyse dauerte 8 Stunden. Biuretreaktion fiel negativ aus.

**Edestin.**

Das kristallinische Präparat ist in Anwendung genommen. 5,0 g des Proteins und 100 ccm Lösung wurden für die Versuche gebraucht. Die Stickstoffverteilung war wie in den anderen Experimenten ausgeführt.

Stärke der Säure	0,5% <sup>1)</sup>	2% <sup>2)</sup>	3% <sup>3)</sup>	5%	8%	10%	15%	15% <sup>4)</sup>
Total N	0,4773	0,8106	0,9184	0,9148	0,9541	0,8562	0,8895	0,9144
N im Filtrat von halbsatur. ZnSO <sub>4</sub> Lösung	0,3786	0,6557	0,8093	0,8227	—	—	—	—
% von N in obiger Lösung	79,74%	80,91%	88,12%	89,93%	—	—	—	—
N im Filtrat von satur. ZnSO <sub>4</sub> Lösung	0,2223	0,4877	0,7084	0,7731	0,9514	0,8156	—	—
% von N in obiger Lösung	46,55%	60,16%	76,61%	84,5%	99,72%	95,2%	—	—
N im Filtrat von P. W. Acid. Präcipitat	0,0110	0,1635	0,3628	0,3858	0,4018	0,3581	0,4491	0,4130
% von N in obiger Lösung	2,03%	20,17%	39,5%	42,17%	42,11%	41,8%	47,43%	45,27%

Eine Übersicht der Tabellen macht es klar, daß Gelatine sich verschieden von anderen Proteinen gegen die spaltende Wirkung von Säuren verhält. Die Gelatosen verschwinden schon bei verhältnismäßig schwachem Angreifen. Weiter merkt man, daß bei der Gelatine eine maximale Ausbeute an Aminosäuren entsteht im Momente, wo die biuretgebenden Substanzen verschwinden. Beim Casein und Edestin bei derselben Phase der Hydrolyse (biuretfrei werden) sind noch nicht alle Aminosäuren freigesetzt, da die Ausbeute an Aminosäuren noch nicht maximal ist. Dieser Umstand macht es wahrscheinlich, daß auch die einfacheren Peptide, die am Aufbau dieser Körper teilnehmen, resistenter sind als jene der Gelatine.

1) Bei dem Auflösen von 5,0 g des Proteins in 100 ccm 0,5 prozentiger Säure bildete sich eine Gallerte, die für die Versuche nicht gebraucht werden konnte. Beim Erwärmen auf dem Wasserbade ging die Substanz in Lösung und schied sich beim Kühlen ein Niederschlag aus. Die Substanz wurde also auf dem Wasserbade 24 Stunden lang und dann im Autoklaven 8 Stunden lang bei 140° C erhitzt. Nach dem Abkühlen wurde der Niederschlag abfiltriert, gewaschen und die vereinigten Filtrate analysiert.

2) 5,0 g Edestin in 100 ccm Säure wurden 24 Stunden lang auf dem Wasserbade und 8 Stunden im Autoklaven bei 140—160° C erhitzt. Beim Abkühlen bildete sich ein Niederschlag. Die vereinigten Filtrate wie oben behandelt.

3) Erhitzt 4 Stunden im Autoklaven. Beim Abkühlen bildete sich ein ganz unbedeutender Niederschlag. Auch die folgenden Hydrolysen wurden durch 4stündiges Erhitzen im Autoklaven bei 140—160° C ausgeführt.

4) Erhitzt 6 Stunden. Die Lösung war biuretfrei.

## Über die tryptische Verdauung des Eialbumins.

Von

P. A. Levene und W. A. Beatty.

(Von dem Rockefeller Institute for medical Research, N. Y.)

(Eingegangen am 1. Mai 1907.)

Die Wirkung verschiedener Protase auf Eiweißkörper war in den letzten Jahren der Gegenstand mehrerer Untersuchungen, doch sind die Resultate, die gegenwärtig erhalten sind, sehr gering im Vergleich zu dem, was aus diesem Studium für die Chemie der Proteine, für die Aufklärung der biologischen Rolle dieser Fragmente und für die Aufklärung der Rolle verschiedener Proteasen im Organismus zu erwarten ist. Die wichtigste Aufgabe unserer Zeit ist unzweifelhaft die Analyse der komplizierteren Fragmente des Moleküls. Ihre Bedeutung für die Proteinchemie ist durch die genialen Arbeiten von E. Fischer kargestellt, und über die physiologische Rolle dieser Körper kann man aus dem Vergleiche des Nährwertes der Produkte, welche durch fermentative und durch Säurespaltung der Proteine erhalten sind, schließen.

Bei der tryptischen Verdauung der Gelatine<sup>1)</sup> ist es einem von uns gelungen, das erste kristallinische Peptid auf analytischem Wege darzustellen. Man dürfte erwarten, daß ähnliche Körper auch bei der Verdauung anderer Proteine entstehen würden. Auch die Auffindung der Ordnung, in welcher die verschiedenen Komponenten des Moleküls abgespalten werden, ist von großem theoretischen Interesse.

Die vorliegende Arbeit wurde mit der Absicht gemacht, die tryptische Verdauung des Eialbumins von diesen Ausgangspunkten zu untersuchen. Dieses Protein war gewählt, weil es durch die

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<sup>1)</sup> Journ. of Exper. Med. 8, 180, 1906.

Erfahrung von Stookey<sup>1)</sup> und einem von uns, von Vernon<sup>2)</sup> und von anderen bekannt geworden ist, daß es der Wirkung des Trypsins einen bedeutenden Widerstand entgegensetzt. Nun erwies sich aber der Widerstand viel größer als erwartet, und aus den kristallinen Produkten konnte nur Leucin, Isoleucin und Tryptophan nachgewiesen werden. Außerdem ist ein Körper erhalten worden, der scheinbar zur Gruppe der Proteinochrome gehört, aber kein Indolderivat ist, und schließlich ein biuret-freier Körper, welcher nach der Säurespaltung nur Lysin und Glycocol lieferte.

1 kg Protein wurde in 12 l 0,5proz. Natriumcarbonatlösung aufgelöst und mit 15,0 g Trypsinum purissimum Grubler vier Monate der Verdauung überlassen. Die Flüssigkeit reagierte nicht mehr alkalisch; sie wurde aufgeköcht unter sorgfältiger Zugabe von Essigsäure.

Der größte Teil des Eiweißkörpers schied sich als Coagulum aus. Das Filtrat wurde zu kleinem Volumen eingedampft. Es bildete sich dabei ein Niederschlag, der scheinbar aus Tyrosin und aus Rohleucin bestand. Das Filtrat von diesem wurde mit konzentrierter Phosphorwolframsäure (4 Teile Säure, 1 Teil Wasser) so lange behandelt, bis noch ein gummiartiger Niederschlag sich bildete. Das Filtrat von diesem wurde von Phosphorwolframsäure befreit und nach Hopkins und Cole auf die Anwesenheit von Tryptophan untersucht. Das Filtrat vom Tryptophanquecksilberniederschlag wurde von Quecksilber und von Schwefelsäure befreit und die Lösung mit Kupferoxyd gekocht. Die Kupfersalze wurden wie in der vorigen Analyse in drei Fraktionen getrennt: eine in absolutem Alkohol löslich, eine in 75% Alkohol löslich und die dritte in Wasser löslich. Jeder von diesen Teilen wurde nach demselben Verfahren, wie in der vorigen Arbeit angegeben ist, ausgeführt, aber es gelang nicht, Prolin oder die anderen Aminosäuren zu erhalten.

#### Die Leucinfraction.

Es wurde versucht, durch fraktionierte Kristallisation aus dieser Tyrosin zu gewinnen; dieses gelang aber nicht. Die Hauptmasse wurde dann mit Phosphorwolframsäure in 4 Teile frak-

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<sup>1)</sup> Journ. of Med. Research 10, 2, 1903.

<sup>2)</sup> Journ. of Physiol. 31, 346, 1904.

tioniert, in der Hoffnung, auf diese Weise die eventuell vorhandenen Aminosäuren zu trennen. In allen Fällen waren aber die Eigenschaften des Leucins so überwiegend, daß es besser schien, sie zu vereinigen und nach dem oben angegebenen Verfahren mit Bleizuckerlösung und Ammoniak zu behandeln, in der Absicht, Leucin von Aminovaleriansäure zu trennen. Die Hauptmassen kamen aber in dem Bleiniederschlag nieder. Die Substanz, von Blei befreit, enthielt bald nach der ersten Kristallisation die folgende Zusammensetzung:

0,1767 g der Substanz gaben bei Verbrennung 0,3572 CO<sub>2</sub> und 0,1578 g H<sub>2</sub>O.

Für C<sub>6</sub>H<sub>13</sub>NO<sub>2</sub>:

Berechnet	Gefunden
C = 54,89%	55,13%
H = 10,01%	9,92%

2 g der Substanz wurden dann zum Inaktivieren im Autoklav mit 30 g Wasser und 8 g Barythydrat 20 Stunden auf 175° C erhitzt. Die Lösung wurde dann von Barium befreit und mit Naphthalinisocyanat nach Neuberg und Manasse behandelt. Das gebildete Hydantoin besaß einen Schmelzpunkt von 179 bis 180° C.

Das Filtrat von Bleileucin wurde von Blei befreit, zur Trockne eingedampft und der Rückstand aus 80% Essigsäure zu kristallisieren versucht; es schied sich aber kein Niederschlag aus. Nach Zugabe eines gleichen Volumens von absolutem Alkohol bildete sich eine kleine Kristallisation, die aber nicht die Zusammensetzung einer reinen Aminovaleriansäure besaß.

0,1690 g der Substanz gaben 0,3309 g CO<sub>2</sub> und 0,1324 g H<sub>2</sub>O.

Für C<sub>6</sub>H<sub>11</sub>NO<sub>2</sub>:

Berechnet	Gefunden
C = 51,22%	53,50%
H = 9,48%	8,75%

### Tryptophanfraktion.

Aus dieser Fraktion ließ sich die reine Substanz in perlmutterartig glänzenden Kristallen, mit allen Eigenschaften des Tryptophans, darstellen.

### Phosphorwolframsäure-Niederschlag.

Diese Fraktion bestand hauptsächlich aus peptonartigen Substanzen, und bei der Untersuchung der Verdauungsprodukte der Gelatine gelang es, aus der ähnlichen Fraktion das Prolinglycylpiperasid zu gewinnen. Es wurde versucht, auch aus dieser Fraktion ähnliche Substanzen zu gewinnen. Zu diesem Zwecke entfernte man die Phosphorwolframsäure und dampfte die Lösung so weit wie möglich ein. Der Rückstand wurde mit absolutem Alkohol ausgezogen; der Auszug zu einem ganz kleinen Volumen konzentriert und mit Aceton gefällt. Das Acetonfiltrat befreite man von Aceton, löste wieder in absolutem Alkohol und behandelte mit absolutem Äther. Beim Verdunsten der alkohol-ätherischen Extrakte, erhalten auf ähnliche Weise aus dem Verdauungsprodukte der Gelatine, erhielt man das Prolinglycil. In dem vorliegenden Versuche gelang es aber nicht, eine kristallinische Masse zu erhalten. Auch war diese Fraktion sowie der alkohol-ätherische Niederschlag nicht biuretfrei. Sie schmeckten aber stark bitter. Nun schien es nicht für unmöglich anzunehmen, daß die Peptide durch anwesende peptonartige Substanzen am Kristallisieren verhindert waren, und man mußte sich bemühen, die biuretgebenden Substanzen zu entfernen. Dieses gelang vollkommen beim Anwenden von Bromwasser. (Letzteres ist kein allgemeines Fällungsmittel für alle peptonartigen Körper. Wir konnten uns wiederholt davon überzeugen, daß die Entfernung aller biuretgebenden Substanzen aus einem Verdauungsprodukte durch dieses Reagens unmöglich war.) Es bildete sich dabei ein schwarzer Niederschlag, der an das schwarze Proteinchromogen der älteren Forscher erinnerte. Es löste sich in schwachen Alkalien, um beim Neutralisieren wieder zu erscheinen, es löste sich auch in Alkohol und mit Amylalkohol kann er mit dunkelbrauner Farbe extrahiert werden. Zur Reinigung wurde die Substanz mit Amylalkohol extrahiert, bei vermindertem Drucke verdunstet und bis zum konstanten Gewicht getrocknet. Die Substanz enthielt keinen Schwefel, entwickelte bei der Verbrennung nicht den Geruch des Indols und Skatols, sondern einen solchen an Phenol erinnernden. Die Substanz hatte die folgende Zusammensetzung:

0,2017 g der Substanz gaben 0,3134 g  $\text{CO}_2$  und 0,0679 g  $\text{H}_2\text{O}$ ;  
C = 42,6%; H = 3,76%.



0,2000 g der Substanz beim Glühen mit Soda gaben AgBr = 0,1198; Br = 25,53%; Br =

0,1740 g der Substanz gaben 14,8 ccm (über 50% KOH) N bei  $T^{\circ} = 24^{\circ} \text{C}$  und  $P = 760 \text{ mm}$ ; N = 9,81.

Die Substanz bestand also wahrscheinlich aus einem verhältnismäßig einfachen Peptide mit basischen Eigenschaften.

Der schwarze Bromkörper von

Kurajeff <sup>1)</sup>	Levene und Beatty
C = 45,15	42,6
H = 2,91	3,76
N = 10,32	9,81
Br = 27,23	25,53
S = 0,90	0

#### Lysin - Glycylpeptid.

Das Filtrat vom schwarzen Niederschlage wurde, um das Brom zu entfernen, mit Chloroform extrahiert, die Lösung bei vermindertem Druck und bei etwa  $60^{\circ} \text{C}$  eingedampft. Die so erhaltene Lösung zeigte keine Spur von Biuret, sie wurde wieder mit einer Phosphorwolframsäurelösung gefällt und der Niederschlag auf übliche Weise mit Baryt von Phosphorwolframsäure befreit. Die Lösung hatte keine Neigung zum Kristallisieren, besaß basische Eigenschaften, schmeckte stark bitter; der erste Gedanke war, daß hier Hexonbasen vorlagen. Es wurden deswegen Vorversuche mit Pikrinsäure und mit Pikrolinsäure gemacht. Es gelang aber mit keinem von diesen Reagentien, einen Niederschlag auch nach längerem Stehen zu erhalten. Die Substanz wurde dann wieder zur Trockne eingedampft, mit absolutem Alkohol aufgenommen und mit Aceton gefällt. Das Filtrat wurde von Aceton befreit und mit Alkohol und Äther extrahiert. Keine dieser drei Fraktionen zeigte Neigung zum Kristallisieren. Die letzte Fraktion wurde dann vom Alkoholäther befreit und im geschmolzenen Rohre 5 Stunden auf  $125^{\circ} \text{C}$  mit konzentrierter Salzsäure erhitzt. Die resultierende Flüssigkeit wurde von Salzsäure mittels Silbersulfat und dann quantitativ von Schwefelsäure befreit. Die Flüssigkeit bei vermindertem Drucke bis zur Trockne eingedampft. Der Rückstand löste sich vollkommen in absolutem

<sup>1)</sup> Zeitschr. f. physiol. Chemie 26, 508, 1899.

Alkohol, und diese Lösung gab einen Niederschlag mit alkoholischer Pikrolonsäurelösung. Das Filtrat hiervon wurde von Pikrolonsäure befreit. In einem Vorversuche konnte man einen Niederschlag mit Pikrinsäure erhalten, als aber die Hauptlösung mit alkoholischer Pikrinsäure versetzt wurde, bildete sich, sogar nach längerem Stehen bei  $-1^{\circ}\text{C}$ , kein Niederschlag. Der Alkohol wurde dann verdunstet und die zurückgebliebene sirupartige Masse mit Wasser aufgenommen. Es bildete sich sofort ein Niederschlag, welcher sich als alkohollöslich erwies. Er wurde auf Saugepumpen abfiltriert, tropfenweise mit Alkohol gewaschen und das rohe Präparat zur Stickstoffbestimmung gebraucht. Die Hauptmasse wurde darauf aus Wasser umkristallisiert. Es schieden sich dabei glänzende Platten aus.

Die Analyse des Pikrolonates ergab die folgenden Zahlen:

0,1633 g gaben 29,0 ccm Stickstoff (über 50% KOH) bei  $T = 21,5^{\circ}\text{C}$  und  $P = 752$ .

0,1767 g gaben 0,3048 g  $\text{CO}_2$  und 0,0838 g  $\text{H}_2\text{O}$ .

Für  $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2 \cdot \text{C}_{10}\text{H}_8\text{N}_4\text{O}_5$ :

Berechnet	Gefunden
C = 46,88%	47,04%
H = 5,36%	5,27%
N = 20,49	20,39%

Es lag also ohne Zweifel Lysinpikrolonat vor. Das aus dem Filtrate dargestellte Pikrat besaß die folgende Zusammensetzung:

0,1690 g der Substanz gaben 0,1972 g  $\text{CO}_2$  und 0,0369 g  $\text{H}_2\text{O}$ .

0,1701 g der Substanz gaben 27,3 ccm Stickstoff (über 50% KOH) bei  $T = 18,0^{\circ}\text{C}$  und bei  $P = 760$  mm.

Für  $\text{C}_2\text{H}_5\text{NO}_2 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$ :

Berechnet	Gefunden
C = 31,58%	31,80%
H = 2,63%	2,39%
N = 18,42%	18,82%

Die Resultate der Hydrolyse waren sehr unerwartet und berechtigten die Annahme, daß ein Lysinglycyl vorlag. Wie bekannt, sind die von Fischer und Suzuky dargestellten Peptide der Hexonbasen nicht in kristallinischer Form erhalten worden.

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## ON THE HISTOGENESIS OF TUMORS, PARTICULARLY CANCER.

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I. *Introduction.*—There exist at present two groups of opinions with regard to the origin of tumors. The first one, purely ætiological, is the parasitic theory; the second assumes primary tissue changes as basis for the development of tumor growth.

The parasitic idea in modern form is extremely limited in application. Even its most ardent exponents have so far only applied it to the carcinomata; but even if this theory (it can hardly be termed one) should gain more ground, it could not demand general recognition as an explanation of the important features connected with the origin and growth of tumors, for it would become necessary to account for different tumors, the mixed growths, the occurrence of two malignant tumors of different type in one individual and even in the embryo, the transitional tumors and, finally, structural differences in individual tumors. All these the parasitic theory fails and will fail to explain. Furthermore, it cannot be understood why a specific parasite should cause certain cells not only to proliferate, but also to assume definite structural characteristics.

This supposition forms, then, from the pathological standpoint at least, no basis for discussion, because, even if everyone should be satisfied as to the occurrence of definite microorganisms in certain tumors, the origin of the tumor itself and its history would still remain entirely obscure.

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The second, much greater, group assumes structural deviations within tissues as primary for tumor growths. It does this by supposing that the cause lies either in changed relation of tissue elements or within the cell from which the tumor originates.

It cannot be the purpose of this study to present a complete historical review of this most productive field of pathological research, but it becomes necessary to outline shortly those of the present ideas of that group which form an introduction to the intelligent understanding of observations presented below.

Beginning with the idea that the origin of a growth is to be found in changed tissue relations, two supplementing theories attract attention; the older and limited theory of embryonic rests of Cohnheim, and the more extensive one of isolated germinal cell groups of Ribbert.

It is, of course, well known that Cohnheim's conception was that misplaced or not misplaced groups of embryonic cells gave origin to tumors; and this applied with considerable force to the congenital and complex tumors. But Cohnheim himself was unable to maintain this idea for the genesis of all tumors, and found especial difficulty in the very explanation of malignant tumors. At the best no idea was conveyed why a misplaced embryonic rest should begin to grow at a definite time of the organism's life, nor were the various types of malignant and transitional tumors at all clear. A much more elaborate and extensive theory, which may be regarded as a continuation of Cohnheim's, was later developed by Ribbert.

Ribbert regards as the cause of all tumors a dislocation or isolation of germinal cell groups not necessarily embryonic, which may take place during uterine or extrauterine life. He does not assume as essential any other specific cause or force, for the growth and proliferation of the tumor cells, than

those which are found in all other physiological processes of growth in the body. It becomes necessary only to produce certain conditions favorable to growth to start the latent and constantly present tendency of cells to proliferate. These conditions are furnished, then, when tissue elements are separated from their physiological continuity.

Ribbert introduces further certain auxiliary hypotheses: Not every isolated germinal cell group leads to tumor growth, but such a cell group must not possess any growth restraining relations which are active in a normal structure. It cannot be arranged as an organ is, and it cannot functionate normally. It cannot contain the growth restraining tissue tension. A certain retrograde change in the cells is admitted by Ribbert as being favorable to the growth, but only secondary and not indispensable.

Lack in internal organization of an isolated germinal cell group is, then, the true basis of tumor growth. If this lack is an incomplete one and also depending upon the original structure of the part, a benign growth will result; but if this lack is complete a malignant tumor originates. Even malignant tumors are organized as such from the start.

Concretely speaking, then, Ribbert does not believe that the epithelium in cancer, for instance, proliferates unless it has become disconnected, isolated from its original situation; and he, therefore, ascribes an important rôle to chronic inflammatory lesions as factors in the isolation of cell groups. There are several theoretical considerations as well as direct observations that make this theory in the main untenable.

Ribbert admits that simple isolation of cell groups is infrequently followed by tumor growth, but resorts here to the lack of internal organization and restraint of tissue tension. However, it would appear that nowhere is such a condition better illustrated than in the healing of wounds, and in the

accidental or willful transplantation of epithelium in such parts. Here, also, isolation of cell groups and a lack of restraint of tissue tension are constant features, but tumors do not ordinarily follow. On the contrary, the tissue assumes here the maturing character from the very start, misplaced groups of cells are consumed and eliminated, the proliferation stops as soon as the loss of substance has been replaced, and this in spite of the fact that previous normal relations have been destroyed and never reestablished. The scar differs necessarily from former physiological conditions, causes changed relations, and disturbs the continuity of tissue; and these conditions prevail wherever healing takes place, be it skin, mucous membrane, or parenchymatous organ. We know that, under certain conditions, foreign cells may reach other organs; placental cells, for instance, reach the lungs or the liver; but ordinarily such misplaced migrated cells do not proliferate nor do they produce growth. Metastatic tumor cells do, however. Other factors must enter into this besides simple isolation, restraint of tissue tension, and normal power of growth.

I. have described, some time ago, a remarkable example of tissue transplantation of well preserved portions of cirrhotic liver, incident to softening of a carcinomatous growth. Well preserved lobules, or parts of them, situated at the periphery of the broken down tumor, had been dislodged by that process, and had been passively carried into the bile ducts with softened tumor masses; from there they had reached the duodenum and the head of the pancreas. Here they had become firmly implanted by cicatricial tissue. In some instances whole lobules with surrounding portal tissues were visible within the pancreas. In these the evidences of cirrhosis with marked bile duct proliferation and jaundice were as plain as in the liver itself. In few a carcinomatous change in the liver cells could be seen

clearly, as in the liver, and in these the surrounding tissue became infiltrated with cancer cells, their cicatricial restriction soon disappeared. In the others, however, a gradual replacement by fibrous, cellular tissue invading the transplanted lobules occurred early; the proliferation of the bile ducts ceased, the whole lobule or group of liver cells became atrophic and ultimately disappeared. It appears that in these transplanted lobules, or portions of them, little or no internal organization was left, and still, in spite of an already existing tendency by the epithelium of the bile ducts to proliferate, the majority of these lobules showed no evidence of beginning tumor growth. Only in those, in which we can assume that the cancerous development had commenced before they had become isolated and reached the pancreas, the tendency to proliferate indiscriminately to overcome the opposing restraint of the cicatricial tissue was manifest.

This remarkable and probably unique instance is mentioned in this connection to show how unlikely it is that a simple isolation of cell groups, even under the conditions favoring proliferation, is sufficient to excite tumor growth.

It may as well be argued that lack of internal organization to which Ribbert is obliged to ascribe so much is the result and not the cause of tumor growth.

In order to explain malignant and benign tumors, Ribbert holds, in the latter, a greater approach to the normal organization of the isolated cell groups. But allowing that the lack of internal organization is the only cause of malignant growths, then once given, it ought to show similar results at all times. We know, however, that this is not the case, even in tumors of the same type, for it is only necessary to recall the great variety of sarcomata and carcinomata and the various forms of mixed malignant growths. Here it would become necessary to look

for factors which determine this peculiar behavior in the development of cells, determine, for instance, a small round cell sarcoma in one case, a spindle, giant, or mixed cell sarcoma in another, and the combination, occasionally observed, of carcinoma and sarcoma in one tumor.

A similar difficulty is experienced with this theory in the understanding of transitional tumors, and in the explanation of the polymorphous appearance of cells in rapidly growing tumors, which frequently show morphological conditions unlike anything ever seen under normal conditions.

This last factor has always been an important point against Ribbert's contention, and has been especially emphasized by those who find the cause of tumor growth in changes in the cell character. Before reviewing shortly that second group of ideas, however, it is well to mention the attempts which have been made to explain the growth of tumors by combining the effects of changes in the cells with changed tissue relations. The old theory of Thiersch, as later followed by Waldeyer and others, assumed that carcinomata developed as a consequence of disturbed relations between connective tissue and epithelium. Thiersch held that in advanced years, as the consequence of nutritive disturbances, the proliferative activity becomes more marked in the epithelium than in the connective tissue, and that the epithelium may grow by substitution into the connective tissue. Weakness on the part of the latter prevents its resistance. More recently Adami has elaborated a theory which has for its basis the idea that, under certain conditions, cells acquire a habit of growth, in which they are aided by a reduction of tissue tension on the cells:

Active cell division and proliferation occurs only in conditions in which the cell cannot fully utilize the assimilative material and the energy stored up in the assimilation of that material in the performance of its



specific function. . . . Such conditions are found in the reduction of tension on cells, and certain energies before necessary to counteract others are free, and thus becoming capable of a diversion of their purpose, or when stimulation from without results in increased assimilation and storage of nuclear and cell material which now from any condition cannot be utilized in the performance of specific functions. . . . The longer the cells are diverted from their extrinsic functions to proliferative activity the greater the momentum acquired, and the habit of growth is set up. Then they may continue to grow in the absence of those conditions which irritated their proliferation in the first place, and we obtain functionless cell growth.

In direct opposition to Ribbert, Hauser attributes the cause of tumors to a fundamental biological change in the cells, and refers principally to the carcinomata. His idea is based on the independence of these epithelial cells and the experimental evidence of transplantation. He regards this biological change as variation in Darwin's sense. Change in the nutritive media influences the energy of life and an excess of nutrition is to be regarded as the most important factor for variability. Such a variation may, then, be found in lessened functional activities and in the predominance of other latent or weak properties.

A sharp definition of those ideas which regard cellular changes as fundamental for tumor development is to be found in von Hanseemann's theory of anaplasia. By this term is meant an entire change in the biological character of the cells, in which they lose their previously developed high differentiation and form an entirely new type of cells, again more closely resembling the undifferentiated ovum. A precedence to this idea was given by a now discarded interpretation of Weissman, of the expulsion of the polar globules from the ovum. He believed that an egg cell which previously held the differentiation of a germinal epithelial cell, became an un-

differentiated egg cell by the expulsion of those chromosones which represent the somatic properties of the cells. Von Hanseemann's idea, however, originated directly from the morphological observation of asymmetric mitoses of cancer cells, which suggested to him that, corresponding to the small nucleus of one of the resulting cells, a similar loss of differentiation might occur. This morphological observation has not stood the test of time as being in any way characteristic of cancer, but the idea of anaplasia has survived as a hypothetical conception. While von Hanseemann insists that anaplasia does not take place in the sense of an embryonic return, and that these cells represent an entity specific to the tumor, this idea is related to the view of older investigators.

For this reason Beneke holds that the cellular change cannot be regarded as a return, but a faulty progression of the blastomatic growth, "an unrestrained progression on the road of protoplasmic development, a cataplasia."

In a very noteworthy communication Marchand has developed these thoughts. He has formed the opinion that a faulty metabolism, which is incident to a degeneration of the cell, precedes tumor proliferation; that as the result of this process cells acquire toxic properties by which they injure the neighboring tissue, and then, unrestrained, freely invade it.

But, to return; according to von Hanseemann, anaplasia itself does not give rise to tumor growth. This requires a definite stimulus. In a normal tissue such a stimulus would only produce hyperplasia, but, in an anaplastic cell, it is followed by malignant tumor growth.

To this Borst remarks that it is an inherent weakness of the theory that it can only account for malignant growths. Beneke, on the other hand, who, as we saw above, regards as the primary factor a pro-

gressive development of vegetative functions, with diminished activity of others, believes that specifically formative irritants, characteristic for individual cells, exist. These, acting in excess, would overthrow physiological and cause blastomatic growth. He returns, therefore, in a great degree to the old idea of formative stimuli. Against all these ideas of stimulative growth Ribbert argues that it is questionable whether growth can ever be attributed to an irritant. For a stimulus acting on a cell can only produce one of two possibilities, functional activity or injury. No stimulus or irritant can ever act on isolated cells, and other tissue changes would then have to be considered. Growth as a result of stimulation, Ribbert holds, can only take place indirectly, by a permanent change in the condition of all the tissues involved.

It will be observed that the ideas of the second group do justice in a much greater degree than those of the first, to the changed morphological character of the tumor cells, the degree of which, it appears, goes hand in hand with the rapidity of growth. These latter theories assume what seems plain to most morphologists, a new cell type which, in a hypothetical way, develops or descends from normal cells. It is in just that point, however, that additional information is needed for a better understanding of this obscure change. I shall present in the following lines some morphological observations, which directly touch upon the change of tissue into tumor cells, and which will allow us to form a more satisfactory conception of the origin of tumor growth, than is possible without a morphological basis.

II. *The Evidencc.*—It can readily be appreciated that an investigation into the early life of tumor growths is associated with considerable difficulty, for it is infrequent to gain possession of tumors in such early stages of their development as

to allow an opinion of the exact genesis of their growth, and especially of the origin of the essential cells composing them. Ribbert and Borst properly draw attention to the misleading pictures obtained in more advanced tumors, and the frequent misinterpretation of such findings. Indeed, it cannot be doubted that many descriptions of the transformations of normal into cancer epithelium represent an invasion from other sources instead of a local change. For the growth, running along in deeper, narrow channels from an original old focus, may suddenly reenter new parts of the parenchyma at some distance and thereby create an impression, at first sight, of a new focus developed in the midst of relatively normal tissue. Such observations are especially frequently made in cancers of mucous membranes, where the glandular loops penetrate the submucous tissues and, advancing there, suddenly reenter the mucous membrane and invade the normal glandular structures. On superficial examination it would appear that we had to do with a new focus, while careful study will establish the true connection.

Out of a number of observations I shall describe in detail one which, in my opinion, offered the most conclusive testimony bearing upon the views expressed below.

The close relationship of tumor growth and chronic inflammation received early attention and consideration, partly from the clinical, but largely from the histological, association. From early times until now this relation has figured seriously in theories of tumors. It gave the impetus to the old conception of formative stimuli and, in the very recent views of Ribbert, inflammatory changes are ascribed an important rôle in the dislocation of cell groups.

It is a matter of not infrequent occurrence to observe, in connection with the more slowly pro-

gressing inflammatory conditions, changes which very closely resemble new growths and even gradually merge into what doubtless presents itself as a tumor. We may refer here only to the bile duct proliferation of the liver in certain forms of cirrhosis, nodular hyperplasia in regenerating liver tissue and in the adrenal bodies, the formation of glandular loops in indurated areas of the lungs, and others. But it has become evident in this study of early malignant growths that inflammatory changes which precede tumors, or are intimately connected with their origin, attain importance in so far as they involve degeneration and atrophy of cells from which the tumor originates. An exceptionally striking illustration was furnished me over two years ago, in a case of primary carcinoma of the liver in a hypertrophic cirrhosis (Hanot's type), and which came to autopsy at a comparatively early stage of development. This case, already published in detail, for which reason I shall only review it here, presented many interesting features, one of which has already been spoken of in connection with the transplantation of tissues.

It was plainly revealed that the origin of the cancer was a transformation of multiple groups of liver cells, sometimes only involving few within one lobule and nearly always centrally located. These microscopic areas, best observed in those parts of the liver which showed, as yet, no gross cancer formation, demonstrated a direct change of atrophic, degenerated, wasting liver cells into cancer cells, while they were still in perfect continuity with each other and still entered into the formation of the lobule. At this very early stage such a lobule showed no other structural changes except those incident to the cirrhosis. I described fully at that time how the cancerous change in these degenerated liver cells occurred. They gradually lost their typical protoplasm, the nucleus became smaller, its

chromatin structure became fainter until finally only little of it remained, with a faint rim of surrounding protoplasm. At this point some of these cells showed a very striking change, in a rapid, irregular production of rich chromatin arranged with much less structural definition than the normal nucleus, and necessarily leading to marked enlargement of it. Intimately associated with the production of this new atypical nucleus was the accumulation of a smooth, supple protoplasm, and an immediate tendency of the nucleus to divide in atypical fashion. I have been able in subsequent studies to detect three well defined stages in this cellular transformation.

The first one, which may be named the *stadium destructivum*, is represented by a cell of the type of the liver cell, with extensive loss and granular degeneration of protoplasm. The nucleus has lost its structural chromatin arrangement and most of it has disappeared. There remains the form of a vesicle, with little or no central chromatin, no distinct nucleolus and, at the periphery, small, rather faint, apparently unconnected, chromatin granules.

The second picture is one in which the nucleus shows marked enlargement with irregular production of small chromatic granules within the vesicle of the same type as in stage 1, but without definite arrangement or connection. A faint nucleolus may be visible. The protoplasm around such nuclei is still of the degenerated, granular type, and much wasted. This may be called *stadium regenerativum incipiens*.

In the third stage, finally, these chromatin granules have largely coalesced to form one to more larger rich clumps which are connected; a pale nucleolus is distinct. At this point an accumulation of smooth, supple protoplasm takes place around the very large nucleus, stamping the cell at once as an entirely different type of cell. This is the *stadium carcinomatosum*.

A new cell type, identical with the cancer cells, was thus formed directly from the degenerated, wasting liver cells, bridged over, only, by a small mass of remaining nuclear chromatin. But these cells still entered at that time into the construction of the lobule, and showed the arrangement in rows after their individual change in cell character had occurred, and no disturbance of the lobule had taken place; ample proof that these small islands were not metastases, but primary cancerous foci. However, as soon as these new cells began to reproduce, they broke with the former physiological arrangement and structure. As they had lost the morphological character as liver cells, they now proved their new functional type by rapid independent growth, with utter disregard to their original source and surroundings. They began to infiltrate, their true malignant nature was manifest, a carcinomatous growth was established.

Not until these cancer cells had proliferated, then, was their connection with each other lost and their independence shown, and it is important to note that the secretion of bile was frequently retained in cells which had, as yet, not lost their continuity with other liver cells, but still entered into the formation of the lobule.

I entertained, at that time, the idea that cancer cells developed from preexisting degenerating parenchyma cells and from retained chromatin portions of the nucleus. This change, as shown by a progressive loss of continuity and ability to secrete bile, was to be regarded as a gradual one, and the cells, thus formed, were morphologically and functionally distinct.

It might be mentioned here that, in spite of an active proliferation of the epithelium of the bile ducts, as commonly found in Hanot's cirrhosis, the duct cells appeared perfectly uninvolved, a fact which, in my opinion, argues against a specific can-

cer stimulus, inasmuch as one would expect cells already proliferating to respond much quicker to such a stimulus than quiescent, degenerating cells.

This case corroborated fully the observations already made by von Heukelom, but frequently contradicted, concerning the multiple origin of cancer of the liver, and the direct transformation of liver cells into cancer cells. It was only a short time later that two publications came to my notice which corroborated Heukelom's and my own observations.

L. Polak Daniels, in an article on the histogenesis of primary carcinoma of the liver, reports multiple, primary, carcinomatous degeneration of epithelial cells in their original situation; and Tolot, in the instance of a cancer of the liver found in a tuberculous man, and without cirrhosis, also observed the direct transformation of liver into tumor cells as follows: "Sur un assez grand nombre de points les cellules néoplastiques se développer non par transformation des cellules normales, mais au contraire en dehors de ses dernières, qui sont peu à peu refoulées et comprimées et disparaissent de la sorte. On voit alors les cellules hépatiques non dégénérées s'allonger, former des travers de plus en plus minces, tandis qu'à leurs côtés les travers des cellules néoplastiques s'étalent, larges et hypercolorées."

III. *Epicrisis*.—This rare instance allowed us to observe very definitely a gradual but direct transformation of degenerating and wasting parenchyma cells into those of a new growth, and by taking origin from a retained portion of nuclear substance. This formation took place, then, on the basis of a far advanced degeneration of the cells and directly from them, the essential features of this degeneration consisting of extensive loss of protoplasm and nuclear chromatin.

Further, the first step in the production of the tumor cells is a nucleus which is made up of structurally undefined, undifferentiated clumps of



chromatin. This nucleus produces later a protoplasm quite unlike that of the ancestral cell.<sup>1</sup> We find, then, that a retained part of the chromatin of the original nucleus forms the connecting link between degenerating parenchyma cells and the resulting cancer cell, and that the tumor cell nucleus shows no such distinction as the normal one. It is to the nucleus, then, that we naturally turn for the explanation of these phenomena.

The experimental observations of O. and R. Hertwig, Klebs, Boveri, Verworn, Minot, and others have clearly demonstrated the intimate relation between nucleus and protoplasm and the essential rôle played by the nucleus and its chromatin in the development and in the metabolism of the cell (*cytomorphosis*, Minot). A definite interchange of substances between nucleus and protoplasm is essential to the healthy life of the cell, and disturbance in either direction is speedily followed by evidences of degeneration. This metabolism of the cell is its real process of life, the phenomena of life only its expression.

We know, for instance, that during glandular activity, augmentation of the chromatin is a constant feature (Stöhr and Garnier). Korschelt has pointed out that, in the secreting nurse cell attached to the eggs of forficula, the peripheral position of the nucleus and its richness in chromatin are undoubtedly correlated with cell metabolism. Carlier found that, in the gland cell of the newt's stomach, the chromatin spreads itself out on the inner surface of the nuclear membrane, and that this condensation is directly connected with the formation of a pro-

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<sup>1</sup> Suggested by Minot's work on cytomorphosis during growth and senescence, observations are now contemplated in this laboratory on the relative size of nucleus and protoplasm in different forms of tumor and stages of tumor growth. In the case which I here describe, the enormous increase of the nucleus in relation to the protoplasm, during the early stage of tumor proliferation, is very plain, and my impression is that this is retained in those cells of tumors which keep up their rapid proliferation. Exact investigations of this point are needed.

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zymogen. In this connection the observations of Gerhardt appear instructive. He found in the glands of rabbits after section of the chorda, focal degeneration of the protoplasm of the cell, and after section of the sympathetic, focal degeneration of the nucleus. It is, therefore, very probable that even the influence of the nerves on secretion is indirectly due to structural changes induced in nucleus and protoplasm, and their necessarily disturbed relations.

The important investigations of Eycleshymer, dealing with the relations of nucleus and protoplasm during growth, and from whose article I quote, have led him to similar conclusions with regard to the rôle of the nucleus and its chromatin in the muscle cell, "they suggest that cellular degeneration and regeneration are accompanied by volumetric structural and chemical changes in chromatin, . . . that nuclear material plays a most important part in cytoplasmic synthesis."

Of very great importance as bearing upon the subject under discussion are the recent observations on the chromidial network and two kinds of nuclei in the lowest animal life.<sup>2</sup>

There has been described by Hertwig structures within the protoplasm of low animal forms which he terms chromidia or chromidial network. They are represented by small bodies or a chromidial network within the plasma which are undoubtedly derived from the nucleus. The chromidial network may form new nuclei or even take their place, from which fact Hertwig concluded its nuclear relations. He termed the new nuclei secondary nuclei. The observations of Schaudinn have thrown considerable light on these findings. He showed that low animal forms possess in their vegetative condition a nucleus

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<sup>2</sup> I owe the knowledge of these references, which appear to be of great importance in the histogenesis of tumors, entirely to Dr. James Ewing, who informs me that he has been working on that subject for some time.

and a chromidial network, and that toward the end of the vegetable life of the organism the vegetative nucleus disintegrates, while the chromidial network forms gamets, is disassociated by marked plasmatic currents, and gives rise to two organisms with re-organizing chromatic network. Such, for instance, is the case in the amœba of dysentery. Toward the end of its vegetative life the nucleus gives rise to plasmatic chromidia forming spores, while it itself disintegrates. In other words, the chromidial network is the *Anlage* of the sexual nucleus, and the vegetative nucleus disappears before propagation.

Now it has been found that the nature of this chromidial network is not a uniform one; in some organisms it seems to stand in close relation to the metabolism, while in others in the same relation to propagation or cell division. We may, therefore, distinguish between vegetative and germinal chromidia.

But, the observations of Schaudinn have demonstrated two kinds of nuclei in the infusoria, sexual or germinal, and vegetative nuclei. In these simplest forms of animal life both of these nuclei appear side by side. In the propagation the germinal nucleus becomes active and, after fertilization, forms a new vegetative nucleus to take the place of the old one. While it had been thought that the occurrence of double nuclei was confined to the infusoria, later investigation has extended this to other forms of protozoan life, and that each of these nuclei is able to appear in the form of chromidia. Those of the vegetative type are known as genuine chromidia, those of the germinal type as sporetia. In some of these forms both kinds of nuclei are present in the form of one or more vegetative nuclei, and in the form of sporetia as propagative nuclei. The condition becomes somewhat more complicated where the separation of both nuclear forms occurs only temporarily for the purpose of sexual propa-

gation. Each nucleus, however, even in these cases functionates and divides. Finally, recognition of both nuclear kinds is most difficult to observe when their separation occurs only shortly before the division, as in the *amoeba coli*.

These results have opened a new field for investigation and Schaudinn properly remarks that it must now be the purpose to trace these two forms of nuclear substance in the metazoan cell. The observations of Goldschmidt make this point very possible for some of the higher developed organisms, for he has been able to establish chromidial networks and their possible relation to nuclear function in the cells of *ascaris*.

The bearing of these investigations on the cytology of higher animal life appears to be this: that the single nucleus of the metazoan cell represents a structure of complex qualitative characters arising from fusion of chromatic substances which, in the lower forms, appear more or less independent of one another.

In view of this knowledge and the morphological observations presented in this paper it seems probable that the various parts of the metazoan nucleus and its chromatin have a direct specific connection with various functions or groups of functions; upon their integrity, definite connection, and proper relations, rests the healthy life of cells. Should, during a degeneration, an unequal loss of nuclear substance and chromatin occur in such a fashion as to retain certain parts of it which are intimately connected with certain functions, while others succumb, it would necessarily allow the remaining portions to control the future of the cell.

I believe that an analogy can be found in certain forms of hypersecretion of parenchymatous organs in which extensive degeneration of the cells is the main feature. There can be no question that the amount of secretion furnished by some of these

organs during late and far advanced destruction of the whole organ is such that it cannot be attributed to a hyperactivity of remaining normal cells. I refer especially to certain forms of inflammation of mucous membranes.

Cases of chronic gastritis in which at autopsy a uniform destruction of the gastric mucous membrane is found while the secretion of gastric juice or mucoid material in very large amounts continues to the time of death, belong to this category. The same is true of the paralytic secretions of watery saliva which continue even after all nerves passing into the gland have been divided. During this process the gland itself gradually diminishes in size, becomes waxlike, and the cells of the alveoli show the resting appearance even to a greater extent than normal resting glands, and undergo atrophy. Still the hypersecretion continues until absolute loss of the glandular structure occurs. Chemical examination of these secretions indicates that they cannot be regarded as a transudation, for they contain principles specific to glandular activity, although the composition of that secretion indicates an unusual or perverse cellular activity.

In view of the experimental and anatomical evidence presented it is not unlikely that this one sided hyperactivity on the part of secreting cells represents an unequal morphological and functional loss incident to a slow degeneration; for the disintegration of the cells in all probability not being a uniform, equally progressing material and functional loss to one degree, retains some better than others. An unequal morphological and functional balance is thereby established, and the life during that slow process of destruction is carried on with functions of unequal strength and importance. Such a disturbed balance, however, leads naturally to a predominance of those functions which have been better retained over those which have been weakened or

entirely lost. Thus it becomes possible during this process that secreting cells may hypersecrete some substance, undoubtedly not as a result of a greater normal functional activity, but as a result of a lost functional balance acquired during their degeneration.

If we extend these thoughts to the problem of tumor formation of cells it would appear possible that, in an extended degeneration and waste of cells which is slow but progressive, the same overactivity may ultimately be taken by the lowest vegetative functions of assimilation and reproduction, when all higher ones are much weakened or abolished. These functions would gradually become more powerful, overrule others and, in some cases, after entire loss of other balancing ones, control cell life entirely.

If these ideas are borne out by further observations they would, I believe, become valuable in the explanation of tumor growths, for they would establish the fact that the tumor represents no embryonic return, no unexplainable biological change in the character of cells, but the direct result of a characteristic degeneration leading to a loss of specific parts of nuclear substance, especially its chromatin.

It is true that the embryonic cell somewhat resembles the tumor cell in its life, for here, also, the function of reproduction seems to control; but it has latent within it other potencies, the ability to develop. As nucleus and protoplasm become more differentiated, the embryonic cell shows its vast difference from the tumor cell. This has gone in the other direction. From a highly differentiated cell it has descended by loss of such structural and functional differentiation to a cell unlike any normal cell. Under no conditions could it develop or regain its former character. Its functions are not latent, as in the embryonic cell, but permanently lost. It is Marchand's great merit to place this as an important point in the distinction of tumor cells from any other young cell.

But, as I have pointed out elsewhere, it is not necessary that in the degeneration of cells allowing a predominance of the reproductive activity, so as to induce tumor growth, all other functions should cease. On the contrary, in the number of remaining functions, and their relations and importance, one would find regularity or irregularity, similarity to the normal or utter change. Thus benign, mixed, and malignant growths result, and the change from the first to the latter becomes plausible.

The close relationship of inflammatory parenchymatous changes to tumor growths in border line cases, where distinct evidences of the tendency to atypical new growth is shown by the formation of different types of cells, and a more irregular arrangement than the normal, while at the same time a restraint in the progress and invasion by the new growth is clear, seem to indicate that instances occur in which a sufficient number of functions are retained within cells to balance and outweigh the reproductive one, and limit its absolute power and control. This, in connection with the opposing influence of the surrounding tissue, would be sufficient to keep the growth from going beyond local manifestations in such cases. This factor may well enter into malignancy and the metastatic power of all tumors. It can also be appreciated how easy it would be, under such conditions, to upset that unstable balance and then have true progressive tumor growth. Herein, in my opinion, lies the close relation between inflammation and tumors.

We find further an explanation for the metastases and infiltration of malignant tumors. Undoubtedly resistance and tension of the surrounding tissue constitute here important factors, but tumor cells have this in their favor: that, as one sided cells they can act in their capacity of growth more strongly than normal multifunctionating cells. Their power is mainly directed along the line of growth

in which they are constantly aided by overproduction.

The disastrous result of their overproduction lies in the very nature of the process. If a hypersecreting cell dies, its work is finished, is lost; the organism is freed from the enemy. Not so in the tumor cell; it is constantly outlived by its own production, new cells. The organism cannot be freed from this form of degenerative hyperproduction.

It is very interesting in this connection to recall that Minot, from the embryological standpoint, especially emphasizes the tendency of mesoblastic tissues to degenerate early in life, while hypoblastic and epiblastic tissues show that tendency much later. We know that mesoblastic tumors are essentially those of youth and epithelial those of later life.

Ribbert opposes the ideas here expressed, as he holds that it would be erroneous to speak of a tumor degeneration of cells, in view of the fact that tumor growth is a progressive process, and that an injury to the cell can never produce growth. But this is a misconstruction of the question at issue, for these views do not assume to regard the tumor growth as a degeneration of the cell, nor that injury itself can ever produce growth, but hold that a characteristic chain of degenerative processes, as outlined above, precedes tumor growth and makes it possible.

It is left undecided whether a specific or any other stimulus is required for the activity of thus established latent forces, nor is this ætiological question of much more interest to the pathologist than the spark as the cause of the explosion is to the physicist. In one as in the other case the true cause must be looked for in inherent properties of the substance.

I am under very great obligations to Professor Minot, of Harvard University, and Professor Ewing, of Cornell University, for important suggestions and helpful references, to Dr. T. C. Jane-



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## EXPERIMENTAL PLEURISY—RESOLUTION OF A FIBRINOUS EXUDATE.

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The purpose of the present study has been to determine the part taken by enzymes in the resolution of a fibrinous exudate. Previous studies have shown that those cells, which with inflammation act as phagocytes and are capable of intracellular digestion, contain two proteolytic enzymes. One enzyme first described by Friedrich Müller is peculiar to the polynuclear leucocytes with fine granulation and is most efficient in the presence of a weakly alkaline medium. The second enzyme, which is present in the mononuclear phagocytes or macrophages, digests only in the presence of acid and resembles the so-called ~~autolytic~~ enzyme which is present in almost all tissues of the body. The action of these enzymes is best studied in exudates produced by sterile inflammatory irritants, since bacteria themselves contain proteolytic enzymes which may be mistaken for the enzymes peculiar to the exudate.

When turpentine is injected into the subcutaneous tissue of the dog an abscess containing thick, yellowish white pus is formed and causes extensive solution of tissue and undermining of the skin. The purulent fluid contains cells in great number many of which undergo disintegration so that the small amount of turbid fluid which by centrifugalization can be separated from the cells of the exudate contains fat droplets and other products doubtless derived from destroyed leucocytes; no fibrin is present. I have previously shown<sup>1</sup> that the serum removed by centrifugalization from this purulent exudate, unlike the serum of the blood, fails to inhibit the proteolytic enzyme contained in the polynuclear leucocytes (leucoprotease).

When an equal quantity of turpentine, one cubic centimeter, is injected into the pleural cavity of the dog, the reaction which re-

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<sup>1</sup> *Jour. of Exper. Med.*, 1906, viii, 536.

sults is very different. Fluid accumulates in large quantity so that at the end of three days there may be one hundred or more cubic centimeters. During the first two or three days after injection this fluid is coagulable, and fibrin in considerable quantity is deposited upon the pleural surfaces. The accumulation of fluid and its gradual disappearance may be followed with considerable accuracy during life by percussion of the animal's chest. Only by such examination during life has it been possible to follow the course of each experiment and to determine when the inflammatory reaction is progressing and when receding.

*Sero-fibrinous Pleurisy.*—In all instances turpentine has been injected into the right pleural cavity. When the dog is standing dullness on percussion caused by the heart extends a variable distance to the right of the median line; occasionally there is at the usual level of the uppermost pair of teats absolute dullness or flatness on percussion one or two centimeters from the mid-line, but in most instances there is no absolute dullness, but merely impairment of resonance (relative dullness) over an area corresponding to the underlying heart two or three centimeters to the right of the mid-line. With accumulation of fluid there is increase of dullness in the dependent part of the chest so that the gradual rise of the upper level of dullness measured from the median line may be determined from day to day. Immediately above the level of absolute dullness is an area of impaired resonance usually one or two centimeters across. By changing the position of the animal so that the vertebral column is upright, there is change in the distribution of dullness on percussion corresponding to change in the position of the fluid, but this change at times occurs slowly and is evident only after the upright position has been maintained for several minutes. In the upright position the area of flatness measured to the right from the median line diminishes, but does not return to the normal limit present before injection of turpentine, and at the same time flatness appears in the back over the lowermost part of the chest next to the vertebral column. Movable dullness is recognizable here when the limit of hepatic dullness has been previously mapped out with the animal in its normal position. Aspiration of fluid has been much facilitated by inserting the

needle in this area of movable dullness and withdrawing fluid while the animal is held in upright position.

By carefully determining by percussion the height of fluid from day to day, it has been found that almost constant changes follow the injection of turpentine. The gradual increase of fluid during three days and its subsequent disappearance is illustrated by the following experiment, in which the height of fluid was determined in the normal horizontal position.

*Experiment I.*

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	2.9 cm.	1 c.c. turpentine injected into right pleural cavity.
After one day	3.1 cm.	4.8 cm.	Animal is inert.
After two days	4.7 cm.	6.0 cm.	Animal appears sick.
After three days	5.5 cm.	7.0 cm.	Animal is somewhat inert.
After four days	4.8 cm.	7.0 cm.	Animal appears to be well.
After five days	0 cm.	6.3 cm.	

The animal has been killed at the end of five days; the right pleural cavity contains 43 c.c. of deep red fluid (containing blood) and over the ventral half of the external surface of the lung is a thin layer of fibrin.

The foregoing experiment serves to indicate the limitations of percussion applied to the dog's chest, for although the cavity has contained forty-three cubic centimeters of fluid there has been no absolute dullness on percussion.

In nine of twelve experiments the maximum height of fluid was present at the end of three days after injection of one cubic centimeter of turpentine into the right pleural cavity. In three instances the maximum amount of dullness was present at the end of two days. Having reached a maximum the dullness disappears with much rapidity. With subsidence of fluid it is noteworthy that the area of impaired resonance often does not diminish as rapidly as the absolute dullness. Examination after death indicates that such impairment of resonance on percussion may be caused by a layer of fibrin still present over the surface of the lung. Moreover, it has been found that even when, at the end of six or more days, fluid has completely disappeared from the cavity resonance near the sternum may still be absent or impaired, because there is in this situation a layer of fibrin of variable thickness. Careful

percussion of the chest during the course of such experiments has the further advantage that the success of attempted intrapleural injection may be determined after one or two days. If the injecting needle has entered the lung a sterile intrapulmonary abscess results and instead of dullness over the dependent part of the chest percussion shows flatness of an especially hard resistant character about the site of injection. By percussion after aspiration it is possible to determine if fluid present in the chest has been almost completely removed.

At the end of two or three days after injection of turpentine, when there is greatest dullness on percussion, the fluid in the pleural cavity may be one hundred cubic centimeters or more. The fluid is usually turbid and pale yellow, but the presence of red corpuscles may give it a reddish color. Cells are present in such small number that after centrifugalization they are found to represent only a very small proportion, usually about half of one per cent. of the total volume of the exudate. At the end of five or six days fluid has in most instances completely or almost completely disappeared from the cavity. The following table shows the quantity of fluid present in the chest at autopsy performed at various intervals after the injection of one cubic centimeter of turpentine.

TABLE I.

Time after Injection	2 Days.	3 Days.	4 Days.	5 Days.	6 Days.	7 Days.
	106 cm.	22 cm.	130 cm.	43 cm.	1 cm.	0.5 cm.
	96 cm.		38 cm.	1 cm.	0 cm.	
			25 cm.		0 cm.	
			7 cm.			

These figures show that the fluid present in the pleural cavity varies considerably in different animals at the same stage of the inflammatory reaction although the same irritant has been injected; percussion of a much larger number of animals has shown that the maximum accumulation of fluid is reached at the end of two or more frequently of three days. The rapidity with which fluid disappears is subject to wide variation though in most instances at the end of five or six days the quantity remaining is trivial.

The intensity of the inflammatory reaction bears no constant relation to quantity of irritant injected. The volume of the fluid

which accumulates in the chest during the first three days after intrapleural injection is roughly indicated by the extent of dullness on percussion, the animals employed being dogs of fairly uniform size weighing four or five kilos. The following experiments are quoted to show the variable effect produced by injection of one half, of one, or of two cubic centimeters of turpentine.

*After Injection of One Half Cubic Centimeter of Turpentine.*—After injection of 0.5 cubic centimeter of turpentine the maximum amount of dullness was present in one animal after one day, in one after two days, in the third after three days. The least (Experiment II) and the greatest (Experiment III) amount of dullness resulting is recorded as follows:

*Experiment II.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	1.1 cm.
After one day	0 cm.	4.8 cm.
After two days	0 cm.	2.6 cm.
After three days	0 cm.	1.1 cm.

*Experiment III.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.4 cm.
After one day	4.3 cm.	5.4 cm.
After two days	5.8 cm.	7.7 cm.
After three days	7.6 cm.	9.2 cm.

*After Injection of One Cubic Centimeter of Turpentine.*—Of ten experiments, in three the maximum amount of dullness was present at the end of two days, in seven at the end of three days. The least (Experiment IV) and the greatest (Experiment V) dullness was as follows:

*Experiment IV.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.2 cm.
After one day	0 cm.	2.2 cm.
After two days	3.8 cm.	5.2 cm.
After three days	0 cm.	3.5 cm.

*Experiment V.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	3.9 cm.
After one day	4.5 cm.	8.2 cm.
After two days	7.0 cm.	8.6 cm.
After three days	7.7 cm.	9.2 cm.

*After Injection of Two Cubic Centimeters of Turpentine.*—In four experiments the maximum of dullness occurred after two days, and in two experiments after three days. Experiments showing the least (Experiment VI) and the greatest dullness (Experiment VII) are as follows:

*Experiment VI.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.0 cm.
After one day	2.9 cm.	5.0 cm.
After two days	4.5 cm.	6.0 cm.
After three days	4.4 cm.	5.4 cm.

*Experiment VII.*

	Absolute Dullness.	Relative Dullness.
Before injection	0 cm.	2.6 cm.
After one day	3.6 cm.	7.0 cm.
After two days	7.2 cm.	10.6 cm.
After three days	10.2 cm.	12.4 cm.

The observations just described show that although there is in general an increase in the amount of exudation corresponding to an increase of the irritant injected, there is such wide variation that the greatest exudation of fluid after injection of one half cubic

centimeter of turpentine may exceed the least exudation caused by two cubic centimeters. Peculiarities of the animal or conditions affecting it obviously have a very important part in determining the intensity of the inflammatory reaction.

Fibrin which forms a layer between the surface of the lung and the parietal pleura is deposited in greatest abundance in the most dependent part of the cavity. The pleural surface of the pericardium and the ventral half of the external surface of the lung, particularly near the sternum, is at the end of two or three days covered by a layer of fibrin a millimeter or more in thickness; whereas over the posterior border of the lung and over the adjacent external surface there is little, if any, fibrin. Everywhere the surfaces of the visceral and parietal pleuræ have lost their normal appearance, and are dull. During the early stages of the inflammatory process the fibrin is opaque white and succulent in appearance, but later, at a time when fluid has disappeared from the cavity, that is at the end of five or six days, fibrin forms a much thinner layer and is dry, gray-white and tough. This layer of fibrin which is usually attached very loosely to the pleura gradually diminishes and at the end of about two weeks or less has completely disappeared. The pleural cavity has become entirely normal or a few organized fibrous shreds represent the only evidence of former inflammation.

Microscopical examination of the fibrin present at the end of two days shows that it is fairly dense in structure, consisting of irregular strands or lamellæ containing imbedded in their substance cells with a single oval or somewhat irregular nucleus and abundant protoplasm. Polynuclear leucocytes are present in considerable number; within the substance of the fibrin they are less numerous than the mononuclear cells but occur in collections of considerable size in spaces between the strands. In scattered areas, on the contrary, the fibrin itself may be infiltrated with polynuclear leucocytes in considerable number. Where fibrin rests upon a serous surface endothelial cells have completely disappeared.

At the end of two days the polynuclear leucocytes which are present are well preserved, the nucleus having the typical multilobed form, but after four or five days both polynuclear and mononuclear cells have in large part disappeared from the fibrin which in places



may be nearly free from cells. Polynuclear leucocytes which remain show various forms of degeneration; the nucleus stains deeply and may be broken into a large number of small round fragments, or may have completely disappeared leaving a necrotic cell recognizable by its size and by comparison with neighboring cells, which have undergone less advanced change. Though the mononuclear cells which at an earlier period have been embedded within the strands of fibrin have in many places disappeared, similar cells are numerous where fibrin is in contact with the underlying pleura. In places occur foci of large mononuclear cells, massed together and situated between strands of fibrin where polynuclear leucocytes were previously most abundant.

At the end of six days organization of fibrin is beginning and blood vessels accompanied by lymphoid and large mononuclear cells in variable number are making their way from the pleural tissue which has often undergone considerable proliferation into the fibrin which in corresponding degree is disappearing. The relation of one or other type of cell to the solution and disappearance of fibrin is not discoverable in sections of the tissue, though not infrequently those large mononuclear cells with abundant protoplasm which act as phagocytes (macrophages) are abundant where fibrin is obviously undergoing solution.

*Pleurisy on the Left Side Caused by Injection into the Right Pleural Cavity.*—The left pleural cavity in most instances remains unaffected by the inflammatory process occurring in the right, but occasionally there is accumulation of fluid containing a small number of leucocytes, and fibrin is deposited especially upon the pleural surface of the mediastinum and of the pericardium and upon the adjacent surface of the left lung. This inflammatory reaction is doubtless referable to irritation of the thin membrane which forms a considerable part of the mediastinum, separating the right and left pleural cavities. In several instances the left pleural cavity has been found to contain a considerable quantity of fluid at a time when fluid has almost completely disappeared from the right cavity which received the irritant. In one instance at the end of four days the right cavity contained only seven cubic centimeters of fluid, whereas the left cavity contained seventy-five cubic centimeters;

in a second experiment at the end of four days the right cavity contained ten cubic centimeters and the left, one hundred and fifty cubic centimeters of fluid; the reaction was at its height in the left pleural cavity at a time when it was subsiding in that cavity which had received the injection.

The sero-fibrinous pleurisy which has been described presents an opportunity to study the accumulation and disappearance of fluid and, as well, the formation and solution of a fibrinous exudate. The whole process of resolution may be studied under conditions uncomplicated by the presence of a living multiplying microorganism which may contain enzymes and other bodies similar to those present in the cells of the inflammatory exudate. Moreover the sero-fibrinous inflammation which is caused by turpentine does not differ in any essential features from similar pleurisies which in man are caused by *Diplococcus lanceolatus* and other microorganisms, the changes which occur reproducing with considerable accuracy the human lesions. The analogy of this type of pleurisy to acute lobar pneumonia is close and resolution in the two processes is in its underlying features doubtless the same.

*Fate of the Irritant.*—In order to determine the relation of the reaction to the irritant the fate of the injected turpentine is of much importance. During the first three days at a time when the fluid is increasing, turpentine can be recognized by its characteristic odor in fluid removed from the chest. At a later period it is no longer recognizable, although in the purulent exudate removed from an abscess caused by subcutaneous injection of turpentine, the substance is recognizable by its odor at a much later period. Accumulation of pleural exudate ceases perhaps when the irritant has been destroyed or removed from the cavity, and subsequently absorption proceeds with variable rapidity, being completed within from four to six days after injection of turpentine.

*Coagulability of the Exuded Fluid.*—When aleuronat in suspension is injected into the pleural cavity of the dog serum containing leucocytes accumulates in large quantity, but fibrin is not deposited upon the pleural surfaces; samples of fluid removed from the chest fail to undergo noteworthy coagulation, and after stand-

ing for a considerable time only a few shreds form about the cells which have subsided. When, on the contrary, turpentine has been used to excite inflammation, fluid removed from the pleural cavity during the first three days after injection of the irritant undergoes such firm coagulation within a few minutes that a small tube containing it may be inverted. The coagulum occupies the entire volume of the fluid. At the end of two days or occasionally after three days coagulation is less complete and the coagulum which forms perhaps after the fluid has stood for a considerable time, occupies only a small part of the entire volume of fluid, whereas in some instances, only a small shred of fibrin may be formed. Fluid removed at the end of five or six days fails to undergo coagulation.

Such casual examination of specimens of fluid removed at various intervals after injection of the irritant suggests that the degree of coagulation is dependent upon the amount of fibrinogen in the fluid and bears no relation to the presence or absence of fibrin ferment. After the third day, at a time when little if any fresh serum finds its way from the blood vessels into the cavity, fibrinogen has been, doubtless, almost completely or completely deposited to form the mass of fibrinous exudate adherent to the pleural surfaces. It is not impossible that this layer of fibrinous deposit is derived from a greater volume of fluid than is present in the cavity at any one time, the serum of the blood forming by weight only from 0.2 to 0.4 per cent. of fibrin. During the period when fluid is accumulating in the cavity it is probable that it is at the same time escaping, in part at least, by way of the lymphatics. Fluid is entering in greater quantity than it is leaving the cavity and that which enters is adding to the fibrinous deposit. After the third day, however, little fluid is entering, while much is absorbed and hence the supply of fibrinogen has ceased.

*Enzymes of the Exuded Fibrin.*—Previous studies<sup>2</sup> have shown that the cells of inflammatory exudates contain two proteolytic enzymes, demonstrable by subjecting coagulable proteid to their action. Proteolysis caused by these enzymes has been measured by estimating by means of the Kjeldahl method the amount of nitrogen of coagulable proteid (heated blood serum) converted by digestion at

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<sup>2</sup> *Jour. of Exper. Med.*, 1905, vii, 316; *ibid.*, 1906, viii, 410.

body temperature into incoagulable form. The activity of enzymes contained in the fibrinous exudate may be tested by a more direct and simple method; disintegration and solution of the exuded fibrin may serve as an index of the activity of the proteolytic enzymes which it contains.

Particles of exuded fibrin, freed from serum by washing in normal salt solution and pressing one or more times between pads of sterile gauze, undergo autolysis when suspended in salt solution (0.85 per cent.). The necessity of removing the serum of the exudate is demonstrated by the following experiment in which measured quantities of serum have inhibited the self-digestion of particles of fibrin of approximately equal size suspended in fluid of which the volume has been brought to twenty cubic centimeters by addition of normal salt solution. Putrefaction is prevented by addition of toluol. The occurrence of digestion as far as it is indicated by disintegration and solution of the particle of fibrin has been indicated by the plus sign (+) and the degree of disintegration by one or more such signs dependent upon the appearance of the fibrin still undissolved.

*Experiment VIII.*

Fibrin of Exudate.	Degree of Digestion after 3 Days at 37° C.
With no serum of exudate	+++
" 0.5 c.c. serum of exudate	++
" 1.0 " " "	+
" 2.0 " " "	0
" 5.0 " " "	0

Under the conditions of the experiment two cubic centimeters of the serum are sufficient to preserve the exuded fibrin unchanged during three days at 37° C., while smaller quantities appreciably inhibit its self-digestion.

The cells of the serous inflammatory exudate produced by injection of aleuronat into the pleural cavity contain two enzymes, one of which, leucoprotease, acts in the presence of an alkaline medium, whereas the other, which for convenience may be designated lymphoprotease, acts only in the presence of acid; lymphoprotease is present in greatest quantity in the exuded cells during the later stages of inflammation. In view of these facts fibrin of the exu-

date present in the pleural cavity at various intervals after injection of turpentine has been subjected to the action of weak acetic acid and of dilute solutions of sodium carbonate. The layer of fibrinous exudate stripped from the surface of the lung is freed from serum and cut into particles of approximately equal size. Each particle of fibrin is suspended in five cubic centimeters of fluid. The test-tube containing fluid and fibrin is tightly closed with a rubber stopper after a small quantity of toluol (five drops) has been added to prevent growth of bacteria. Since the serum of the blood inhibits the action of the enzymes contained in the exudate, one of the equivalent particles of fibrin is for control suspended in five cubic centimeters of fluid containing one cubic centimeter of blood serum; since no digestion occurs and the particle of fibrin remains unchanged, comparison will show the amount of solution in the other tubes. All of the tubes have been incubated at 38° C. during from four to seven days.

In the presence of a weak alkaline solution, fibrin may quickly swell and undergo conversion into a viscid, semi-fluid substance, which is, in part, dissolved in the overlying fluid. Weak acid causes the fibrin to contract and break up into a fine, powder-like sediment. In either case, when digestion occurs, the fluid becomes turbid, while in absence of digestion, the fluid about the unchanged fibrin remains almost clear. Slight digestion may be indicated by erosion of the fibrin recognizable only by comparison with the control suspended in diluted blood serum.

Further evidence of proteolytic digestion may be obtained by testing for incoagulable digestion products the fluid overlying what remains of the fibrin. The simple method of testing for peptones suggested by Obermeyer<sup>3</sup> and employed by Starling,<sup>4</sup> Halliburton and Colls,<sup>5</sup> and others has been used, the tube containing blood serum again serving as control. Three cubic centimeters of the fluid overlying the remains of the fibrin are mixed with an equal quantity of a ten per cent. solution of trichloroacetic acid. After the mixture has been shaken and allowed to stand for a short time, the precipitate

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<sup>3</sup> *Med. Jahrbücher*, 1888, iii, 375. *Ref. Maly's Jahresbericht*, 1889, xix, 7.

<sup>4</sup> *Jour. of Physiol.*, 1893, xiv, 131.

<sup>5</sup> *Jour. of Path. and Bact.*, 1896, iii, 295.

of coagulable proteid is removed by filtration. The filtrate is now tested for biuret reaction. The intensity of the reaction, which has been used by Starling for quantitative estimation, gives indication of the relative amount of peptone and albumose present. Fluid from the control tube uniformly fails to give the reaction, though it contains the native albumins of the serum, whereas, when digestion has occurred, an intense reaction is present.

The following experiment illustrates the character of the digestion which occurs in different media and the method of recording the changes.

*Experiment IX.*

Exuded Fibrin was Allowed to Digest During 7 Days at 37° C.	Fibrin.		Biuret Reaction.	
	Character of Change.	Relative Amount of Solution.	Character of Reaction.	Relative Intensity of Reaction.
With 1 % acetic acid.	Swollen and gelatinous.	?	Negative.	o
With 0.5 % acetic acid.	Dissolved save small amount of sed.	++++	Deep pink.	++++
With 0.2 % acetic acid.	Dissolved save very small amount of sed.	++++	Moderately deep pink.	++++
With neutral reaction.	Slightly eroded.	+	Trace of pink.	+
With 0.2 % sod. carb.	Slightly eroded.	+	Pale pink.	+
With 0.5 % sod. carb.	Slightly eroded.	+	Pale pink.	+
With 1 % sod. carb.	Translucent but intact.	o	Negative.	o
With 1 c.c. blood serum.	Unchanged.	o	Negative.	o

A close parallel exists between the degree of digestion recognizable by naked eye examination of the particle of fibrin and that indicated by the biuret action after precipitation of coagulable proteid by trichloroacetic acid. The method employed serves to distinguish simple solution from digestion. It is well known that fibrin of the blood is dissolved by certain salts and by acids; Fermi<sup>6</sup> found that 0.5 hydrochloric acid dissolved but did not peptonize fibrin. The foregoing experiment shows that the exuded fibrin contains an enzyme which dissolves fibrin and converts it into peptone in the presence of weak acetic acid. In stronger acetic acid (one per cent.) the character of the change is wholly different; the fibrin being swollen and broken into almost transparent particles which nearly fill the entire volume of fluid, it is impossible to determine how much solution has occurred. That acid of this strength is

<sup>6</sup> *Zeit. für Biol.*, 1891, xxxviii, 229.

unfavorable for the action of the enzyme contained in the exuded fibrin is shown by the absence of reaction for peptone. With weaker acid, favorable to the action of the enzyme, absolute parallel between macroscopic solution and peptone reaction cannot be expected, for proteid may be converted by continued action of enzyme into decomposition products which do not give a biuret reaction. The following table shows the behavior of fibrin removed from the chest at intervals of from two to ten days after injection of turpentine, when suspended in media differing in reaction :

TABEL II.

Experiment.	No. of Days After Injection.	With 2 per cent. Acetic Acid.		With Neutral Reaction.		With 2 per cent. Sodium Carbonate.	
		Solution of Fibrin.	Biuret Reaction.	Solution of Fibrin	Biuret Reaction.	Solution of Fibrin.	Biuret Reaction.
A	2	++++	+++	++	+	+	+++
B	2	+++		+		++	
C	3	++++	++++	+	+	+	+
D	4	++++		o		++++	
E	5	++++	++++	+	+	+(?)	+(?)
F	6	++++	+++	o	o	o	o
G	6	+++	++	o	o	o	o
H	6	++++	++++	o	o	o	o
I	9	++++	++++	+++	+	o	o
J	10	+++	+++	o	o	o	o

The table shows that digestion is much less active in a neutral medium than in acid or in alkali. During the first four or five days digestion occurs both in the alkaline and in the acid medium, but at the end of five days the power to digest in the presence of alkali has been lost, so that subsequently little, if any, digestion occurs, save in the presence of acid. It is noteworthy that the ability to undergo digestion in alkali disappears at a time when fluid has undergone almost complete absorption. This relation is not constant, and Experiment G is an exception, the pleural cavity containing sixty-eight cubic centimeters of fluid, in large part encapsulated.

*Injection of Leucocytes into the Inflamed Pleural Cavity.*—In two experiments which follow the ability of exuded fibrin to undergo self-digestion in the presence of alkali has been increased by injecting into the inflamed chest polynuclear leucocytes in considerable quantity.

*Experiment X.*—Sterile pus, obtained four days after injection of turpentine into the subcutaneous tissue of the dog has been injected into the pleural cavity two days after the onset of inflammation.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	3.2 cm.	1 c.c. of turpentine injected into the right pleural cavity.
After 1 day	7.7 cm.	12.5 cm.	Animal is sick.
After 2 days	12.2 cm.	13.9 cm.	14 c.c. pus injected into the right pleural cavity.
After 3 days	16.2 cm.	17.4 cm.	Animal is sick.
After 4 days	13.2 cm.	15.8 cm.	Animal is sick.
After 5 days	11.6 cm.	14.8 cm.	Animal is sick.

The animal has been killed by hæmorrhage under ether. The right pleural cavity contains 175 c.c. of blood-stained fluid. Fibrin is present in large quantity and forms a layer often two millimeters in thickness. In places, this fibrin is grayish, tough and firm, but elsewhere it has a yellowish-white color, and is soft and ragged, as if undergoing disintegration.

Fibrin freed from serum was subjected to autolysis under the conditions previously described.

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	0	+
With 0.2 per cent. sodium carbonate	+ +	+ + +
With 1 c.c. blood serum	0	0

As a result of the injection of fluid containing polynuclear leucocytes in immense number fibrin, removed from the chest five days after the onset of inflammation, digests itself almost as readily in alkali as in acid. Comparison with Table II shows that power to digest in alkali has been augmented. It is, however, not improbable that the pus which was injected two days after the onset of pleurisy contained some turpentine, and, therefore, tended to prolong the effect of the turpentine originally brought in contact with the pleura. To avoid this possibility leucocytes from a purulent exudate were washed before injection.

*Experiment XI.*—Cells from a sterile, purulent exudate obtained by subcutaneous injection of turpentine were separated from the fluid of the pus and washed by centrifugalization in 0.85 per cent. salt solution; after removal of the supernatant fluid, the cells were injected into the inflamed pleural cavity, three days after the onset of pleurisy.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	3.9 cm.	1 c.c. of turpentine was injected into the right pleural cavity.
After 1 day	4.5 cm.	8.2 cm.	Animal sick.
After 2 days	7.0 cm.	8.6 cm.	Animal sick.
After 3 days	7.7 cm.	9.2 cm.	14 c.c. washed leucocytes were injected into the pleural cavity.
After 4 days	0 cm.	3.5 cm.	



When the animal was killed by hæmorrhage under ether, the right pleural cavity contained 45 c.c. of deep red serous fluid. Fibrin formed a thick layer which was soft, yellowish-white and succulent. It is not improbable that a part of the injected turpentine entered the substance of the lung, for a small tear was found at the site of injection. Autolysis of fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	0	+
With 0.2 per cent. sodium carbonate	+ + +	+ + +
With 1 c.c. blood serum	+ (?)	+

It is noteworthy that the injection of washed leucocytes was followed by no increase of fluid indicated by percussion, as in the preceding experiment, and very marked diminution of dullness was found on the following day. The relative ability of fibrin to digest in the presence of alkali is greater than that indicated by any of the experiments recorded in Table II. Leucoprotease is, indeed, so abundant that one cubic centimeter of blood serum has failed to inhibit its action. Microscopical examination of the soft fibrin which is apparently undergoing disintegration shows that it is infiltrated with polynuclear leucocytes in immense number and suggests the probability that these are in large part leucocytes which have been injected into the cavity, since fibrin at a corresponding interval, after simple injection of turpentine, contains relatively few polynuclear cells.

*Anti-Enzymotic Action of the Exuded Fluid.*—Since two stages of enzymotic activity are recognizable during the course of the inflammatory reaction, one requiring the presence of alkali, and the other of acid, it is necessary to determine if conditions present in the fluid of the exudate are such that during life these enzymes are brought into action. The serum of the blood and of the exudate furnish the necessary alkalinity for that enzyme which digests in the presence of alkali, and is characteristic of the polynuclear leucocytes. This enzyme is, however, inhibited by the anti-enzymotic activity of the exuded serum. At no time during the course of the fibrinous inflammation caused by turpentine is this anti-enzymotic action of the fluid surrounding the fibrin lost, and, indeed, only when the corresponding enzyme has disappeared from the fibrin has absorption of fluid removed the influence of the anti-enzyme.

The following experiments show that there is no noteworthy diminution of the anti-enzymotic activity of the exuded fluid when tested with leucoprotease, the enzyme of the polynuclear leucocytes. In each experiment twenty milligrams of dry powder, prepared from polynuclear leucocytes, was allowed to act upon heated blood serum during five days at 37° C., and the amount of digestion was determined by the Kjeldahl method;<sup>7</sup> the ability of the exuded fluid to inhibit the activity of the enzyme was determined by comparing this digestion with that which occurred when the same amount of enzyme acted upon the same amount of coagulated proteid in the presence of a half cubic centimeter of the fluid to be tested. These determinations have been made with the assistance of Miss Bertha I. Barker. By "age of the exudate" should be understood the number of days between injection of turpentine and withdrawal of fluid from the chest either by aspiration or after death. The approximate degree of inhibition has been estimated by subtracting the figure (in cubic centimeters of  $\frac{1}{10}$  N. sulphuric acid) representing digestion in the presence of serum from that representing unrestrained digestion.

TABLE III.

Experiment.	Age of Exudate in Days.	Control.	Digestion with 20 mgr. Leucoprotease.	Digestion with 20 mgr. Leucoprotease and 0.5 c.c. Serum.	Degree of Inhibition.
<i>A</i>	1	2.4 c.c.	16.4 c.c.	5.45 c.c.	10.95 c.c.
	3	2.4 c.c.	16.4 c.c.	6.1 c.c.	10.3 c.c.
<i>B</i>	3	2.1 c.c.	18.9 c.c.	8.85 c.c.	10.05 c.c.
<i>C</i>	5	2.0 c.c.	18.85 c.c.	7.05 c.c.	11.8 c.c.

The following experiment is scarcely necessary to show that fibrin which has been found to digest only in the presence of acid and hence contains only lymphoprotease fails to undergo autolysis in the presence of the exuded serum. The fibrin and serum were obtained from a dog (Table II., Experiment E) five days after injection of turpentine. Table II. shows that this fibrin underwent almost no autolysis in the presence of alkali. The volume of fluid was brought to five cubic centimeters by addition of normal salt solution.

<sup>7</sup> *Jour. of Exper. Med.*, 1906, viii, 410.

*Experiment XII.*

	Solution of Fibrin	Biuret Reaction.
With 0.5 c.c. of exudate serum	0	0
With 1 c.c. of exudate serum	0	0
With 2 c.c. of exudate serum	0	0

The foregoing study shows that the fluid exuded into the chest as a result of the presence of turpentine is coagulable during the first two days of the inflammatory reaction and deposits upon the pleural surfaces a layer of fibrin containing entangled in its substance an immense number of leucocytes. These cells undergo disintegration and set free an enzyme which is capable, in the presence of an alkalinity equal to that of the serum, of dissolving fibrin and other proteid. The serum of the exudate contains an anti-enzyme which in part holds in check this enzyme and is doubtless efficient in limiting its action, thus perhaps protecting injured tissues from destruction. It is difficult to prove that these enzymes are capable of attacking and destroying living tissue, but no facts that are known exclude this possibility. Where polynuclear leucocytes are massed together in the meshes of the compact fibrinous network which forms the layer overlying the lung, enzyme is, perhaps, unrestrained by anti-body, and partial digestion of fibrin results. That such digestion actually occurs is indicated by the appearance of peptone which, absent at the beginning of the process, is present in greatest quantity when, after three days, the inflammatory reaction has reached a maximum.

*The Occurrence of Products of Proteolytic Digestion in the Exuded Fluid.*—Products of proteolysis have been found in fluid withdrawn at intervals after injection of turpentine. By precipitating the albumin and globulin contained in the exuded serum with trichloroacetic acid as recommended by Starling, no biuret reaction is obtained with the filtrate at the end of the first day. At the end of the second day no reaction, or only a trace of reaction occurs, but at the end of the third day, when the quantity of fluid in the chest is greatest, a well marked reaction is usually obtained. At the end of four and of five days a reaction is usually obtainable, but it is less intense than after three days. The following table shows the result of tests made at various intervals after injection of turpentine:

TABLE IV.

*Reaction for Peptone and Albumoses.*

Experiment.	1 Day.	2 Days.	3 Days.	4 Days.	5 Days.
<i>A</i>	o	<i>T</i>	++	+	
<i>B</i>				o	
<i>C</i>	o		+++		+
<i>D</i>		o			
<i>E</i>	o	(?)	o	o	
<i>F</i>				+	

The absence of reaction for peptone is indicated by zero (o). The occurrence of a biuret reaction after coagulation of proteids other than peptone is indicated by the signs of addition, the intensity of the reaction in a given experiment being indicated by from one to three such signs. A trace of peptone causing a scarcely perceptible biuret reaction is indicated by (*T*).

Since peptone is absorbed with much rapidity from the chest, the presence of a well marked reaction at the end of three days indicates that formation of proteolytic digestion products has reached a maximum and now exceeds their absorption.

The following experiment was undertaken with the purpose of determining what influence products of digestion by leucoprotease exert upon the progress of inflammation caused by turpentine. It shows with what rapidity such products are absorbed from the inflamed chest.

*Experiment XIII.*—Products of digestion were obtained by allowing 50 mgr. of leucoprotease, with addition of toluol, to act during four weeks at 37° C. upon 25 c.c. of a mixture of dog's blood serum and normal salt solution, previously heated to 75° C.; 10 c.c. of this fluid sterilized and freed from toluol by heating to 65° C. during one hour were twice injected into the right pleural cavity. This fluid diluted 1:10 (*i. e.*, a dilution probably greater than that produced by the fluid in the chest) gave an intense reaction for peptone.

	Absolute Dullness.	
Before injection	2.3 cm.	1 c.c. turpentine injected into the right pleural cavity.
After 1 day	4.0 cm.	10 c.c. fluid containing digestion products injected. One half hour later fluid withdrawn gave well marked reaction for peptone.
After 2 days	7.8 cm.	Only faint reaction for peptone obtained. 10 c.c. fluid with digestion products again injected.
After 3 days	11.4 cm.	No reaction for peptone.
After 4 days	7.5 cm.	Very faint reaction for peptone.
After 7 days	7.3 cm.	No reaction for peptone.

After the animal had been killed, the pleural cavity was found to contain 25 c.c. of reddish serous fluid, together with an unusually large quantity of fibrin.

*Alkalinity of the Inflammatory Exudate.*—Since an alkaline reaction is essential for the action of one enzyme of the exudate, and an acid reaction for the other, the reaction of the pleural exudate has been studied. At no time does the alkalinity of the exuded fluid disappear, but throughout the course of the inflammation it is less than that of the blood serum. The alkalinity of the exudate was tested by the method proposed by Engel<sup>8</sup> and modified by Gamble.<sup>9</sup> One cubic centimeter of the exudate to be tested was withdrawn with antiseptic precautions from the chest during life, or was obtained at autopsy immediately after death. The exudate was diluted with one hundred times its volume of distilled water and neutralized with  $\frac{1}{100}$  N. sulphuric acid, lacmoid paper being used as indicator. Acid was added until a distinctly acid reaction was obtained, and since acid inadvertently added in excess cannot be accurately neutralized, duplicate determinations were made. With the first specimens alkalinity was determined with a fair degree of accuracy, though usually a slight excess of acid was added. In testing the second specimen, it was possible to use great care in adding acid when the quantity indicated by the first test was approached. Hence the second determination, which has been the smaller, is the more accurate, and is given in the following table, which shows the alkalinity of the exudate determined from two to five days after injection of turpentine. The alkalinity of the blood, which was in a number of instances tested simultaneously, varied for one cubic centimeter from 9.3 to 11 c.c.  $\frac{1}{100}$  N. acid.

TABLE V.

*Alkalinity of Exudate in Cubic Centimeters of 1-100 N. Sulphuric Acid.*

Experiment.	1 Day.	2 Days.	3 Days.	4 Days.	5 Days.
A	—	5.4	—	4.95	—
B	—	—	5.5	—	—
C	—	—	—	—	4.2

At the end of three days after the onset of inflammation, polynuclear leucocytes cease to migrate from the blood vessels into the pleura, and those which are enmeshed in the fibrin undergo necrosis

<sup>8</sup> *Berl. klin. Woch.* 1898, xxxv, 308.

<sup>9</sup> *Jour. of Path. and Bact.*, 1906, xi, 124.

and disappear. At the end of five or six days, fibrin no longer contains that enzyme which is peculiar to the polynuclear leucocytes; fibrin, therefore, fails to disintegrate in the presence of weak alkali, but readily dissolves in acid. Though partial solution of fibrin doubtless referable to the enzyme of the polynuclear leucocytes, has occurred within the chest during the first five days, resolution is not complete, and at the end of the first week of inflammation a thin layer of fibrin still covers the pleural surface of the pericardium and anterior surface of the lung near the median line, and in less quantity is present between the base of the lung and the diaphragm.

*Effect of Carbon-Dioxide on the Enzyme Present in Exuded Fibrin.*—Evidence which has already been discussed shows that the fibrin which remains unresolved by leucoprotease contains an enzyme which acts only in the presence of weak acid; nevertheless fluid in the chest, which is much less alkaline than the blood and diminishes slightly in alkalinity with the progress of inflammation, at no time approaches a neutral or acid reaction. At a later period, when fluid has disappeared from the chest, fibrin is still moistened by lymph which has an alkaline reaction, and though the fibrin contains a proteolytic enzyme capable of digesting in an acid medium, conditions favorable for the action of such an enzyme are at first sight wholly wanting. Nevertheless, the possibility has suggested itself that carbon-dioxide may play the part of an acid, and in the body afford conditions favorable to the slow solution of fibrin.

The following experiments have shown that carbon-dioxide may replace the acetic acid previously used to bring in action the enzyme which alone is present during the second stage of the inflammatory reaction caused by turpentine.

*Experiment XIV.*—Fibrin was obtained from an animal which had received two days before one cubic centimeter of turpentine into the pleural cavity. This fibrin underwent auto-digestion in the presence both of acid and of alkali, but was only slightly altered when suspended in physiological salt solution. Particles of fibrin were suspended in normal salt solution and through the fluid carbon-dioxide was passed during one half hour. The test-tube containing the fluid was still filled with carbon-dioxide when tightly closed with a rubber stopper after addition of a few (five) drops of toluol. The volume of fluid in each tube was five cubic centimeters, normal salt solution being added to make this volume.

	Digestion of Fibrin.	Biuret. Reaction.
With 0.2 per cent. acetic acid (5 days at 37° C.)	+ + + +	
With salt solution alone (5 days at 37° C.)	+	
With 0.2 per cent. sodium carbonate (5 days at 37° C.)	+ + +	
With 1 c.c. blood serum (5 days at 37° C.)	o	
With salt solution alone (2 days at 37° C.)	o	+
With salt solution and carbon-dioxide (2 days at 37° C.)	+	+ +
With salt solution alone (4 days at 37° C.)	+	
With salt solution and carbon-dioxide (4 days at 37° C.)	+ + +	

In the following experiment fibrin which digested only in the presence of acid was used. The experiment repeats and confirms Experiment XIV and demonstrates, moreover, that carbon-dioxide brings this enzyme into action, even though blood serum (one cubic centimeter) is present.

*Experiment XV.*—The fibrin employed was obtained from the pleural cavity of a dog, nine days after the injection of turpentine. Digestion occurred during seven days at 37° C.

	Digestion of Fibrin.	Biuret Reaction.]
With 0.2 per cent. acetic acid	+ + + +	+ + + +
With 0.2 per cent. sodium carbonate	o	o
With normal salt solution	+	o
With salt solution and carbon-dioxide	+ + +	+ +
With salt solution and 1 c.c. blood serum	o	o
With salt solution, 1 c.c. blood serum and carbon-dioxide	+ + + +	+ +

The following experiment confirms those which have just been described, and again shows that in the presence of a small quantity of blood serum (half of a cubic centimeter) active proteolysis occurs when fibrin which digests only with acid is exposed to the action of carbon-dioxide.

*Experiment XVI.*—Fibrin was obtained from the pleural cavity of a dog ten days after the injection of turpentine.

	Digestion of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid (5 days at 37° C.)	+ + +	+ + + +
With 0.2 per cent. sodium carbonate (5 days at 37° C.)	o	o
With salt solution (5 days at 37° C.)	o	o
With 1 c.c. blood serum (5 days at 37° C.)	o	o
With 0.5 c.c. blood serum (2 days at 37° C.)	o	o
With 0.5 c.c. blood serum and carbon-dioxide (2 days at 37° C.)	+	+ +
With 0.5 c.c. blood serum (6 days at 37° C.)	+ +	o
With 0.5 c.c. blood serum and carbon-dioxide (6 days at 37° C.)	+ +	+ + +

The foregoing experiments, which show that carbon-dioxide can bring into action the enzyme remaining in a fibrinous exudate after disappearance of leucoprotease, suggest that carbon-dioxide may favor resolution of fibrin remaining in the pleural cavity during the latter stage of pleurisy caused by turpentine and doubtless by other agencies. Some plausibility is given to this hypothesis by the observations of Ewald,<sup>10</sup> who found that the carbon-dioxide tension of an exudate increases with its age. He studied the pleural exudate from fourteen cases of pleurisy and found that the total volume of carbon-dioxide which could be withdrawn from the exudate with Pflüger's pump after the addition of acid varied from 29.54 to 63.84 per cent., the higher figure being obtained when the exudate had been present in the chest during a considerable length of time. In purulent fluid which had collected quickly and contained leucocytes in great abundance, carbon-dioxide was present in much smaller quantity, whereas the smallest figures (7.92 and 8.05 per cent.) were obtained with pus from abscesses.

#### CONCLUSIONS.

Fibrinous pleurisy produced by a sterile inflammatory irritant offers opportunity for study of the part taken by enzymes of leucocytes in the resolution of a fibrinous exudate. When turpentine is injected into the subcutaneous tissue of the dog, an abscess results, but when an equal quantity of turpentine is injected into the pleural cavity, there is abundant exudation of coagulable fluid and the serous surfaces are covered by a layer of fibrin. Accumulation of fluid which can be followed during life by percussion of the animal's chest reaches a maximum at the end of three days, and then gradually subsides, so that at the end of six days, in most instances, the cavity contains no fluid. Fibrin, though diminished in amount at the time when fluid has been absorbed, is still present, and gradually disappears; at the end of two or three weeks the cavity has returned to the normal, save for a few organized adhesions.

Turpentine injected into the right pleural cavity may cause sero-fibrinous pleurisy on the left side; this inflammation may reach a

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<sup>10</sup> *Ref. Maly's Jahresbericht* 1874, iv, 421.



maximum intensity at a time when pleurisy on the right side is subsiding.

During the early stage of inflammation fibrinous exudate, freed from the serum by washing in salt solution, undergoes digestion when suspended in an alkaline (0.2 per cent. sodium carbonate) or in an acid medium (0.2 per cent. acetic acid). At the end of five days, at a time when fluid is disappearing from the pleural cavity, digestion fails to occur in an alkaline medium, but occurs with much activity in the presence of acid.

During the first stage of the inflammatory reaction, when fluid is abundant and the fibrin which is present digests in alkali, thus indicating the presence of leucoprotease, polynuclear leucocytes are very numerous in the meshes of the fibrin. In the second stage, the exuded fibrin contains only one enzyme digesting in the presence of acid. At this time polynuclear leucocytes have disappeared and only mononuclear cells are embedded in the fibrin.

Products of proteolytic digestion, namely, peptone and albumose, absent in the exuded fluid during the first day or two days of inflammation, are present after three days and are found in less quantity at a later period.

The exuded fluid does not at any stage of the inflammatory reaction lose its power to inhibit both enzymes contained in the leucocytes.

The exudate remains alkaline throughout the period of inflammation, but its alkalinity is less than that of the blood and diminishes slightly with the progress of inflammation.

Since the acids, which in vitro favor the action of the enzyme, present alone during the second stage of the inflammatory reaction, do not occur in the body, the possibility has suggested itself that carbon-dioxide brings this enzyme into action. If carbon-dioxide is passed through normal salt solution in which strips of such fibrin are suspended, digestion is greatly hastened. The normal inhibition exerted by blood serum upon the enzyme is overcome by carbon-dioxide and in the presence of a small quantity of blood serum, carbon-dioxide causes greater enzymotic activity than in the presence of salt solution alone.



THE TRANSFORMATION OF SERO-FIBRINOUS INTO  
PURULENT PLEURISY.

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## THE TRANSFORMATION OF SERO-FIBRINOUS INTO PURULENT PLEURISY.

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The preceding study has shown that turpentine (one cubic centimeter) injected into the pleural cavity of the dog causes rapid exudation of a large quantity of coagulable fluid. Fibrin containing a considerable proportion of the leucocytes which migrate from the blood vessels as a result of the chemiotactic action of the irritant is deposited upon pleural surfaces and the serous fluid about this fibrin contains only a small number of leucocytes. In the fluid is an antienzyme which holds in check the enzyme contained in the polynuclear leucocytes, so that resolution of fibrin proceeds slowly, and at no time does the exudate assume the appearance of pus. The pleural surfaces undergo no permanent alteration, and at the end of about two weeks, the pleural cavity has in most instances become normal, save for a few organized adhesions.

When the same quantity of the inflammatory irritant is injected into the subcutaneous tissue, it is not diluted by a large quantity of fluid. A considerable volume of fluid, perhaps with the aid of the negative pressure within the thorax, readily collects in the relatively spacious pleural cavity, whereas accumulation of fluid in the subcutaneous tissue is checked by the increase of tension which occurs when the tissue is infiltrated with fluid and leucocytes. Examination of this pus has shown<sup>1</sup> that leucoprotease of the polynuclear leucocytes so far exceeds anti-body contained in the serum of the pus that the entire exudate undergoes autolysis when placed under suitable conditions.

If the essential difference between the suppurative inflammation of the subcutaneous tissue with solution of tissue, on the one hand, and the sero-fibrinous pleurisy, with which for a considerable time fibrin remains undissolved, on the other hand, is the relative

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<sup>1</sup> *Jour. of Exper. Med.*, 1906, viii, 536.

proportion of enzyme and antibody, it is probable that sero-fibrinous pleurisy may be converted into empyæma either by increasing the quantity of enzymes or decreasing the quantity of anti-enzyme. The following experiments indicate though they do not conclusively demonstrate that a sero-fibrinous may be converted into a purulent pleurisy by either of the methods which have been mentioned; nevertheless they exhibit very clearly, I believe, the mechanism by which the transition occurs.

If from one half to two cubic centimeters of turpentine are injected into the pleural cavity, turpentine is recognizable by its odor in the pleural exudate until the end of about three days, at a time when accumulation of inflammatory products has reached a maximum. By subsequent injections of the same irritant, there is continued exudation of fluid and emigration of leucocytes. A second injection of turpentine, three days after the first, increases the quantity of exuded fluid and prevents the disappearance of leucoprotease from the fibrin.

*Experiment XVII.*—One cubic centimeter of turpentine was injected into the right pleural cavity of a dog, and three days later the same quantity was again injected.

	Absolute Dullness.	
Before injection	1.2 cm.	1 c.c. turpentine injected.
After 1 day	2.9 cm.	
After 2 days	5.5 cm.	
After 3 days	7.0 cm.	Animal sick; 1 c.c. turpentine again injected.
After 4 days	14.8 cm.	Animal very sick; dyspnœa.
After 5 days		Animal found dead.

The right pleural cavity contains 165 c.c. deep red serous fluid. Loosely attached to the surface of the lung and to the diaphragm is a layer of soft yellowish-white fibrin measuring 3 or 4 mm. in thickness. At the upper part of the sternum is a walled-off cavity containing 5 c.c. of purulent fluid. The left pleural cavity is normal.

The ability of the fibrin present to undergo autolysis after incubation during seven days in various media is indicated by the following table.

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+	++
With neutral reaction	+	++
With 0.2 per cent. sodium carbonate	++++	++++
With 1 c.c. serum of exudate	+	+

\* Biuret reaction was tested with filtrate obtained after precipitation of coagulable proteid by trichloroacetic acid (see description of methods in preceding article).

It is noteworthy that power to digest in an alkaline medium indicating the presence of leucoprotease is far greater than that observed in any of the experiments recorded in Table II of the preceding article. Furthermore, one cubic centimeter of the exuded serum fails to inhibit autolysis.

In the following experiment two injections of turpentine have produced a typically purulent exudate at the end of seven days after the first injection.

*Experiment XVIII.*—The animal received 0.5 c.c. turpentine in the right pleural cavity and three days later an equal quantity.

**Absolute Dullness.**

Before injection	0 cm.	0.5 c.c. turpentine injected.
After 1 day	4.3 cm.	Animal sick.
After 2 days	5.8 cm.	Animal inert.
After 3 days	7.6 cm.	Animal apparently well; 0.5 c.c. turpentine injected over base of right lung.
After 4 days	7.6 cm.	Animal very sick; dyspnœa; area of dullness about site of injection at base of right lung.
After 5 days	9.5 cm.	As before; very marked dyspnœa.
After 7 days	10.7 cm.	As before; killed by hæmorrhage under ether.

The right pleural cavity contained 245 c.c. of fairly thick opaque white purulent fluid. After centrifugalization, cells are found to represent 35 per cent. of the volume. Within the cavity is a voluminous mass of ragged, often semi-solid, yellowish-white fibrin which is evidently in process of disintegration. The surface of the lung is opaque and greenish-white in color.

The serum obtained by centrifugalization of the pleural exudate gives a well-marked reaction for peptone after coagulation with trichloroacetic acid. Agar-agar inoculated with pleural exudate remains sterile.

Digestion of the disintegrating fibrin is indicated as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+ + + +	+ + + +
With 0.2 per cent. sodium carbonate	+ + + +	+ + + +
With 0.5 c.c. serum of exudate	+ + +	+ + +
With 1 c.c. serum of exudate	+ +	+ +
With 2 c.c. serum of exudate	+ + + +	+ + + +
With undiluted serum of exudate	+ + + +	+ + + +

The serum of the exudate increases rather than diminishes the power of the disintegrating fibrin to undergo digestion.

In the following experiment, three injections of turpentine have caused an intense purulent inflammation.

*Experiment XIX.*—A primary injection of 2 c.c. turpentine was followed by two injections of 0.5 c.c. after three and four days respectively.

	Absolute Dullness.	Relative Dullness.	
Before injection	0 cm.	2 cm.	2 c.c. turpentine injected into right pleural cavity.
After 1 day	2.9 cm.	5 cm.	Sick.
After 2 days	4.5 cm.	6 cm.	Sick.
After 3 days	4.4 cm.	5.4 cm.	Apparently well; 0.5 c.c. turpentine injected.
After 4 days	2.7 cm.	3.8 cm.	Apparently well; 0.5 c.c. turpentine injected.
After 6 days		12.4 cm.	Sick; marked dyspnoea.

When the animal was killed at the end of six days, the pleural cavity was found to contain 181 c.c. of opaque creamy-white pus (sterile), which after standing became viscid and on centrifugalization failed to separate into a cellular and non-cellular layer. This accumulation of pus was approximately limited to the anterior two thirds of the pleural cavity, the surface of the abscess cavity being covered in large part by a layer of yellowish-white succulent fibrin. Occupying the remainder of the cavity and situated over the dorsal part of the lung and between lung and diaphragm was a mass of loose fibrin containing in considerable quantity encapsulated serous fluid.

The enzyme content of (a) the fibrin in contact with the purulent exudate and (b) the œdematous fibrin at the base of the lung were tested separately.

(a) *Fibrin in contact with purulent exudate.*

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	++	+++
With neutral reaction	++++	+++
With 0.2 per cent. sodium carbonate	++	++++

(b) *œdematous fibrin at base of lung.*

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+++	++++
With neutral reaction	++	+++
With 0.2 per cent. sodium carbonate	0	+

The absence of very marked increase of absolute dullness, although a large quantity of fluid has been present within the pleural cavity, is perhaps explained by the fact that fibrinous adhesions have held the lung in contact with the chest wall; at the end of six days, there has been, however, impairment of resonance over a considerable part of the chest wall. These facts make it probable that the second and third injections have been confined to that part of the cavity which has been found at autopsy to be the site of purulent exudate. The succulent yellowish-white fibrin in this part of the cavity has undergone digestion with much greater activity in an alkaline than in an acid medium, whereas the fibrin which contains serous fluid more closely resembles that which is present at a corresponding time after a single injection of turpen-

tine, that is, when resolution is in progress; the latter autolyses with much greater activity in the presence of acid than of alkali.

In the preceding article, it has been shown that a single injection of from one half to two cubic centimeters of turpentine is followed by a sero-fibrinous pleurisy which almost constantly undergoes complete recovery with restoration of the pleural cavity to normal. In no instance among thirteen experiments in which examination has been made from three to seven days after injection, and among four experiments of longer duration, has generalized suppuration occurred within the pleural cavity as the result of a single injection of turpentine, though in two instances small macroscopic collections of leucocytes have been found below the pulmonary or parietal pleura. Since these two experiments show that foci of suppuration occur though rarely under the conditions which have been mentioned, their description will precede that of similar lesions which were found far more commonly after the removal of fluid.

#### *Experiment XX.*

##### *Absolute Dullness.*

Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	3.7 cm.	Sick.
After 2 days	5.4 cm.	Sick.
After 3 days	5.9 cm.	Apparently well; killed.

The right pleural cavity contained 22 c.c. deep red serous fluid (sterile). Over the ventro-external surface of the right lung was a layer of white succulent fibrin, 1 to 1.5 mm. in thickness. Upon the surface of the lung within the fibrin were two slightly raised opaque yellow nodules about 3 mm. across. Microscopical examination shows a central mass of polynuclear leucocytes in various stages of degeneration with nuclear fragmentation and necrosis. In a peripheral zone, mononuclear cells with fairly abundant protoplasm and round deeply staining nuclei are numerous.

#### *Experiment XXI.*

##### *Absolute Dullness.*

Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	3.6 cm.	Inert.
After 2 days	4.7 cm.	Sick.
After 3 days	5.5 cm.	Inert.
After 4 days	4.8 cm.	Apparently well.
After 5 days	0 cm.	Relative dullness still present over considerable area.

The right pleural cavity contained 43 c.c. deep red serous fluid. Over the ventro-external surface of the lung was a layer of firm gray-white fibrin, 0.5 to 1 mm. in thickness. Between two lobes was a very small (about 2 mm. across)



collection of creamy white fluid, and upon the parietal pleura, near the apex of the lung, was a low elevation (about 7 mm. across). Microscopical examination of the latter shows a sharply localized cavity filled with polynuclear leucocytes, many of which have undergone degeneration; a smaller number of large and small mononuclear cells are present.

When, on the contrary, fluid is removed by aspiration at the height of exudation, localized pleural abscess not infrequently results. At this time, the cavity contains abundant fibrin in the meshes of which are polynuclear leucocytes in great number. Experiments *in vitro* have shown that this fibrin contains leucoprotease in such quantity that autolysis quickly occurs when the influence of the exuded serum is removed. It is probable that a similar process occurs when fluid is removed from the pleural cavity. The occurrence of abscess will depend upon the completeness with which fluid is withdrawn. In some instances, the aspirating needle was inserted about three centimeters from the sternum, the animal being held with the body in the normal horizontal position; withdrawal of fluid is, perhaps, more complete in this position. In other instances, the limit of hepatic dullness in the back beside the vertebral column was determined with the animal in its natural position. When the animal was placed with its body erect, fluid subsided to the dependent part and a zone of movable dullness was definable in the back above the level of the diaphragm. The aspirating needle was inserted within this area, three or four centimeters to the right of the vertebral column.

In seven experiments all the fluid obtainable was withdrawn from the inflamed pleural cavity two days after injection of the irritant, and in five instances, localized abscesses resulted. In the two experiments which follow, abscess formation failed to occur.

#### *Experiment XXII.*

##### *Absolute Dullness.*

Before injection	0 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	2.9 cm.	
After 2 days	4.9 cm.	50 c.c. straw colored fluid aspirated.
After aspiration	0 cm.	
After 3 days	0 cm.	

The animal was killed at the end of seven days. The right pleural cavity contained no fluid; the entire surface of the right lung, save the posterior border, was covered by an irregular firmly adherent layer of tough white fibrin, in places one millimeter in thickness. Microscopical examination shows organization of fibrin; the fibrin remaining contains mononuclear cells but no polynuclear leucocytes.

*Experiment XXIII.*

## Absolute Dullness.

Before injection	0 cm.	1 c.c. turpentine injected into right pleural cavity.
After 2 days	12.7 cm.	60 c.c. serous fluid aspirated.
After aspiration	5.2 cm.	
After 3 days	5.1 cm.	
After 4 days	5.1 cm.	
After 10 days	3.0 cm.	Animal killed.

The right pleural cavity contained no fluid; abundant tough white fibrin united the anterior aspect of the lung to the chest wall. Microscopical examination shows advanced organization of fibrin containing only cells of mononuclear type.

In three experiments which follow, small abscesses were found after withdrawal of fluid.

*Experiment XXIV.*

## Absolute Dullness.

Before aspiration	2.6 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	3.6 cm.	
After 2 days	4.4 cm.	14.5 c.c. of serous fluid withdrawn.
After aspiration	3.3 cm.	
After 4 days	3.3 cm.	Animal killed.

The right pleural cavity contained 1 c.c. of red turbid fluid. There was much fibrin forming a layer over the ventral aspect of the lung and over the adjacent pericardium. Upon the parietal pleura at the posterior part of the cavity was a yellowish elevation which contained purulent fluid. Within an interlobular cleft upon the anterior margin of the lung was a small enclosed cavity containing thick fluid of purulent appearance.

Microscopical section of fibrin taken at random shows degenerating leucocytes forming collections of considerable size. A section of the nodule upon the parietal pleura shows a well defined abscess cavity within the layer of fibrin; polynuclear leucocytes with nuclear degeneration are mingled in great number with both large mononuclear cells with vesicular nucleus and small cells with deeply staining nucleus.

Autolysis of fibrin from the surface of the lung occurred as follows:

	Solution of Fibrin	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + + +	+ + + +
With neutral reaction	0	0
With 0.2 per cent. sodium carbonate	0	0
With 1 c.c. blood serum	0	0

*Experiment XXV.*

## Absolute Dullness.

Before inoculation	1.3 cm.	1 c.c. of turpentine injected into right pleural cavity.
After 2 days	12.2 cm.	85 c.c. straw colored serous fluid withdrawn.
After aspiration	3.0 cm.	
After 3 days	1.5 cm.	
After 6 days	1.5 cm.	
After 9 days		Killed with ether and bleeding.

The right pleural cavity contained no fluid. Fibrin was abundant, forming a layer about 1 mm. in thickness over the surface of the ventral half of the external surface of the lung. The right lung was bound in places to the pericardium, diaphragm, and parietal pleura. The left pleural cavity was normal.

Within a layer of fibrin between the two lobes of the lung is a small flattened cavity containing viscid fluid of purulent appearance. Microscopical examination shows a central mass of necrotic material containing polynuclear leucocytes in various stages of degeneration. Surrounding it is a zone of large mononuclear cells, many of which have ingested the altered polynuclear leucocytes. A zone of fibrin separates these cells from a thick capsule of newly formed connective tissue.

#### *Experiment XXVI.*

	Absolute Dullness.	
Before inoculation	2.1 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	4.6 cm.	
After 2 days	6.2 cm.	43 c.c. reddish serous fluid removed.
After aspiration	0 cm.	
After 3 days	2 cm.	Sick.
After 4 days	2 cm.	Sick.
After 9 days	3.5 cm.	Apparently well; killed.

The right pleural cavity contained no fluid. The entire upper lobe was bound by a layer of fibrin, from 0.5 to 1.5 mm. in thickness, to the chest wall and pericardium. Within this fibrin was a small flattened cavity, about 1 cm. across and containing thick whitish fluid having the appearance of pus. Corresponding to this focus of softening, fibrin is found by microscopical examination to have undergone solution; polynuclear leucocytes undergoing degeneration are fairly abundant, but similar cells which are necrotic and without nuclei occur in great number.

In the following experiments, aspiration of all the fluid obtainable has been followed by formation of cavities of considerable size containing sero-purulent or purulent fluid, and this localized suppuration has occurred even though the quantity of fluid withdrawn has been relatively small (10 to 15 c.c.). It is not improbable that fluid has been withdrawn from a part of the chest completely or wholly separated by fibrinous adhesion from the remainder of the cavity.

#### *Experiment XXVII.*

	Absolute Dullness.	
Before inoculation	0 cm.	1 c.c. turpentine into right pleural cavity.
After 2 days	7.7 cm.	All fluid (serous) obtainable withdrawn.
After aspiration	2.8 cm.	
After 3 days	4.2 cm.	Sick.
After 4 days	2.5 cm.	Killed.

Within the right pleura walled off by fibrin is a cavity containing 10 c.c. of yellowish purulent fluid; fibrin lining the cavity is soft and ragged and evidently

in process of disintegration. Microscopical examination shows disintegrating fibrin infiltrated with polynuclear leucocytes in immense number. The remainder of the pleural cavity contains about 15 c.c. of reddish serous fluid (sterile on attempted culture), together with a layer of compact gray-white fibrin, 1.5 mm. in thickness. Autolysis of this fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+	+ +
With 0.2 per cent. sodium carbonate	0	0
With 1 c.c. blood serum	0	0

#### *Experiment XXVIII.*

	Absolute Dullness.	
Before injection	1.1 cm.	1 c.c. turpentine injected into right pleural cavity.
After 1 day	5.2 cm.	Sick.
After 2 days	7.5 cm.	All fluid (serous) obtainable withdrawn.
After aspiration	4.5 cm.	Apparently well.
After 4 days	4.3 cm.	
After 8 days	4.2 cm.	Apparently well; killed.

In contact with the sternum and displacing the upper lobe of the right lung is a localized pleural abscess containing 40 c.c. of thick creamy white pus. Attempted inoculation of agar-agar has given a negative result. The remainder of the pleural cavity is normal.

The foregoing experiments have suggested the probability that a sero-fibrinous pleurisy of greater intensity might be uniformly converted into empyæma by withdrawal of fluid. In view of the variable results obtained after a single injection of various amounts of turpentine, a second injection, performed at a time when the inflammatory reaction produced by the primary injection had reached its height, was believed to offer the best promise of uniformly increasing the intensity of the reaction. Experiments XVIII and XIX have shown that such double injection may result in suppuration. But in a part of the experiments suppuration has failed to occur. In such cases, after aspiration of all of the fluid which could be obtained within twenty-four hours, a serous has become a purulent exudate. This result is especially noteworthy because the withdrawal of fluid removes from the chest the greater part if not all of the inflammatory irritant, namely turpentine, which is recognizable by its odor in the aspirated fluid.

*Experiment XXIX.*

	Absolute Dullness.	
Before injection	0 cm.	2 c.c. turpentine into right pleural cavity.
After 1 day	2.3 cm.	
After 2 days	4.1 cm.	
After 3 days	5 cm.	0.5 c.c. turpentine injected.
After 4 days	5.6 cm.	38 c.c. turbid serous fluid (sterile) containing by volume 0.5 per cent. of cells.
After aspiration	0 cm.	

The animal was killed twenty hours after aspiration. The right pleural cavity contained a large quantity of sero-purulent fluid in which were 6.5 per cent. of cells. Fibrin was abundant upon the surface of lung, pericardium, and diaphragm. The basal part of the layer of fibrin, which was usually 2 or 3 mm. in thickness, was fairly firm, but the superficial part was soft, opaque, yellow and partially disintegrated.

Autolysis of fibrin occurred as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. of acetic acid	+ + + +	+ + +
With neutral reaction	+ + + +	+ +
With 0.2 per cent. sodium carbonate	+ + + +	+ + + +

*Experiment XXX.*

	Absolute Dullness.	
Before injection	0 cm.	1 c.c. turpentine into right pleural cavity.
After 1 day	4.4 cm.	
After 3 days	4.8 cm.	1 c.c. turpentine again injected.
After 4 days	9.5 cm.	Withdrawn by aspiration 112 c.c. red serous coagulable fluid (sterile) containing 0.25 per cent. cells; serum gives faint reaction for peptone.
After aspiration	0 cm.	Large zone of relative dullness on percussion.
After 5 days	7.9 cm.	Withdrawn for examination 9 c.c. creamy white purulent fluid (sterile) containing 22 per cent. of cells; serum gives marked reaction for peptone.
After 6 days	14.2 cm.	Sample of thick purulent fluid withdrawn; cells form 52 per cent.; marked reaction for peptone.
After 11 days	16.6 cm.	Animal moribund.

The right pleural cavity contains 400 c.c. thick cream-white pus containing 44.5 per cent. of cells. The parietal pleura is much thickened and the surface of the lung has a greenish-gray color. Adherent to the upper lobe of the lung, and in places elsewhere, is a layer of altered fibrin, soft, friable, and with the appearance of necrotic material.

*Experiment XXXI.*

	Absolute Dullness.	
Before injection	0 cm.	0.5 c.c. turpentine injected into right pleural cavity.
After 1 day	4.5 cm.	
After 2 days	9.7 cm.	
After 4 days	8.2 cm.	0.5 c.c. turpentine again injected.

	Absolute Dullness.	
After 5 days	15.6 cm.	Withdrawn by aspiration 100 c.c. reddish serous fluid containing 0.5 per cent. of cells; faint reaction for peptone.
After aspiration		Over an area about 4 cm. across, absolute dullness remained, but had disappeared in the most dependent part of the chest.
After 7 days	4.5 cm.	Impaired resonance throughout right chest. For examination was withdrawn 1 c.c. of yellowish purulent fluid (sterile) containing approximately 50 per cent. of cells.

At the end of eight days, when the animal was killed, the right pleural cavity contained 168 c.c. of purulent fluid (sterile) with 29 per cent. of cells. The right lung was covered by a mass of fibrin about 5 mm. in thickness, yellowish-white, very soft and evidently in process of disintegration. The serum obtained by centrifugalization of the purulent fluid gives a well marked reaction for peptone (+ +).

Autolysis of the disintegrating fibrin occurred after 7 days at 37° C. as follows:

	Solution of Fibrin.	Biuret Reaction.
With 0.2 per cent. acetic acid	+ + +	+ + +
With neutral reaction	+ + +	+ + +
With 0.2 per cent. sodium carbonate	+ + +	+ + + +
With 1 c.c. blood serum	o	o
With 1 c.c. serum of exudate	+ +	+ +
With 5 c.c. serum of exudate	+ + +	+ + +

In Experiments XXIX and XXX, the fluid present in the pleural cavity as the result of two injections of turpentine has been serous in character at the end of four days after the first injection; aspiration of all the fluid obtainable after puncture at the posterior and basal part of the chest, the animal in upright position, has been followed within twenty-four hours by formation of a purulent exudate. The fluid which was previously thin and reddish, containing less than one per cent. of cells has been replaced by thicker, opaque, white fluid containing, in one instance 6.5 per cent., and in the other 22 per cent. of cells. Fibrin, which with sero-fibrinous inflammation is firm, and grayish-white, has become yellowish-white, and undergoing disintegration is soft or even semi-solid. The fluid part of the exudate which at the time of aspiration exhibited a faint reaction for the products of digestion, twenty-four hours later gives a well-marked reaction. At a later period, the purulent characters of the exudate are intensified, cells becoming more abundant. In Experiment XXXI, examination of the pleural con-

tents has not been made until forty-eight hours after aspiration, and at this time, the cavity has contained fairly thick pus. In Experiment XXX, in which purulent fluid has remained in the chest during six days, fibrin has, in large part, undergone solution.

In order to define the essential difference between a fibrinous and a purulent exudate, tests of the autolytic power of the disintegrating fibrin obtained in the preceding experiments are especially significant when compared with similar tests made with fibrin from a sero-fibrinous exudate (Table II of preceding article); autolysis in an alkaline medium occurs in experiments just described with far greater activity.

In Experiment XXXI, one cubic centimeter of blood serum has completely inhibited digestion of fibrin, yet the same quantity of serum from the purulent exudate has failed to prevent action of the enzyme contained in the fibrin, whereas in the presence of undiluted serum of the exudate, digestion has been more active than with one cubic centimeter, the volume of fluid, after addition of normal salt solution when necessary, being in every instance five cubic centimeters.

The foregoing experiments have been described to illustrate the transformation of sero-fibrinous into a purulent exudate, and to indicate the associated underlying changes which distinguish the latter from the former. They are not believed to indicate that aspiration of fluid in cases of sero-fibrinous pleurisy is a dangerous procedure, changing perhaps a relatively mild pleurisy into empyæma; in the last three experiments which have been described, it is probable that death would have occurred as the result of compression of the lungs and perhaps of the heart at an earlier period, had not aspiration been performed. If they offer any suggestion applicable to treatment of pleurisy, it is that complete withdrawal of fluid with acute pleurisy is undesirable.

These experiments bring further evidence that with an inflammatory reaction which has not reached the degree of intensity indicated by suppuration, leucoprotease of the polynuclear leucocytes is held in check by antienzyme of the exuded serum. Since the pleural cavity presents conditions favorable for the accumulation of fluid, the inflammatory irritant is in the first place rapidly di-

luted; after a singly injection of turpentine, coagulable fluid, together with polynuclear leucocytes rapidly accumulate, but at no stage of the reaction does enzyme overcome the anti-enzyme.

Polynuclear leucocytes which are present within the layer of fibrin deposited upon the surface of the lung removed in part from influence of the serum cause partial solution of this fibrin, but within five days after the onset of pleurisy produced by a single injection of the irritant undergo autolysis and disappear. After aspiration of fluid, two processes may occur, in some instances, side by side. Withdrawal of fluid may, it appears, hasten autolysis and disappearance of polynuclear leucocytes, so that fibrin removed from the chest four days after the onset of pleurisy (Experiments XXIV and XXVII) may fail to digest in the presence of alkali, failure to digest indicating absence of leucoprotease. In association with this fact, it is noteworthy that after aspiration of fluid, resolution of fibrin is incomplete, and at the end of ten days or two weeks, fibrin is much more abundant when fluid has been withdrawn than when pleurisy has pursued an interrupted course.

It is by no means improbable that the localized abscesses which have been found more frequent after aspiration than with uninterrupted pleurisy are referable to a similar cause, namely, increased autolysis no longer restrained by anti-enzyme of the exuded fluid. Enzyme set free by autolysis of cells, on the one hand, may be rapidly removed or destroyed, whereas, on the other hand, when such products are present in large quantity or when their escape is prevented, a localized focus of suppuration, characterized by solution of fibrin, may result. In this way, probably, are formed the localized abscesses which occur when fluid is withdrawn after a single injection of turpentine. Nevertheless, though focal abscesses have been more frequent after aspiration, they occur when the experimental pleurisy has an uninterrupted course.

Increase of enzyme may be equally efficient in transforming a fibrinous into a purulent exudate. For even though a large quantity of fluid accumulates in the chest after repeated injection of the irritant, continued migration of leucocytes, many of which undergo destruction, will finally set free enzyme (leucoprotease) in quantity sufficient to overcome the large amount of anti-enzyme;



that there is a certain quantitative relation between enzyme and anti-enzyme has been shown in a previous publication.<sup>8</sup> The exuded fluid assumes the character of pus; leucocytes are present in immense number and fibrin infiltrated with leucocytes undergoes disintegration.

The effect of a single injection of turpentine varies within such wide limits that in some instances, doubtless dependent upon peculiarities of the animal injected, one half a cubic centimeter may produce more intense inflammation than two cubic centimeters. The effect of repeated injections is equally variable. In some instances, a sero-fibrinous pleurisy results (Experiments XVII, XXIX, XXX and XXXI). It is noteworthy that removal of fluid is followed by transformation of such a sero-fibrinous into a purulent exudate, even though a large part, if not all of the turpentine which still remains in the chest is removed with the aspirated fluid. The serum of the purulent exudate fails to inhibit in vitro the enzyme contained in the fibrin, now infiltrated with an immense number of polynuclear leucocytes. Nevertheless in interpreting the experiments which have been described it must be recognized that a sero-fibrinous inflammation cannot in the transitional stages be sharply separated from suppuration.

There can be little doubt that the purulent exudate in which enzyme is more powerful than anti-enzyme itself acts as an inflammatory irritant and intensifies the exudation of fluid and emigration of leucocytes. When after aspiration of fluid and removal with the fluid of all or almost all of the inflammatory irritant, suppuration fails to occur, or is limited to a small abscess cavity (Experiments XXII to XXVI), there is little or no reaccumulation of fluid. With the occurrence of suppuration, on the contrary, there is rapid accumulation of fluid, which gradually increases in quantity and become richer in cells. Immediately after aspiration, if the explanation which has been offered is correct, the disproportion between enzyme and anti-enzyme is greatest, and there is greatest opportunity for solution of fibrin. The unrestrained enzyme, at the same time has an opportunity to exert an injurious influence upon the pleural surfaces.

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<sup>8</sup> *Jour. of Exper. Med.*, 1906, viii, 536.



ON EXTRACELLULAR AND INTRACELLULAR VENOM  
ACTIVATORS OF THE BLOOD, WITH ESPECIAL  
REFERENCE TO LECITHIN AND FATTY ACIDS  
AND THEIR COMPOUNDS.

By HIDEYO NOGUCHI, M.D.

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## ON EXTRACELLULAR AND INTRACELLULAR VENOM ACTIVATORS OF THE BLOOD, WITH ESPECIAL REFERENCE TO LECITHIN AND FATTY ACIDS AND THEIR COMPOUNDS.<sup>1</sup>

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### INTRODUCTION.

Snake venom forms a hæmolytic compound with pure lecithin when it is shaken with a chloroform solution of this substance.<sup>2</sup> The same is true of the venom of bee.<sup>3</sup> Judging from this important discovery, linked with the fact that the serum as well as the corpuscles of blood yield upon hot alcoholic extraction a large quantity of lecithin, Kyes was led to deduce that the venom activating substance of blood is lecithin. This theory has, however, some rather serious difficulties in explaining certain fundamental phenomena which are observed in venom hæmolysis. In spite of the fact that all serums or corpuscles do yield upon extraction nearly uniform amounts of lecithin, certain kinds of serums possess no activating property; and the susceptibility of the corpuscles of different bloods exhibits a wide variation according to the species of animals. In some instances the corpuscles are completely refractory to the action of venom. Speaking quantitatively there is no relation between the amount of lecithin and that of the venom activating substance of serum or corpuscles. If we are forced to hold lecithin responsible for venom hæmolysis in normal serum or blood corpuscles we must assume that lecithin exists in different states in different bloods, the explanation adopted by Kyes to account for the phenomena observed.

This hypothesis appears to furnish an easy solution of the phenomena, but it requires, in the meanwhile, its verification by experi-

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<sup>1</sup> Received for publication April 15, 1907.

<sup>2</sup> Kyes, *Berl. klin. Woch.*, 1903, xi, 956, 982.

<sup>3</sup> Morgenroth and Carpi, *Berl. klin. Woch.*, 1906, xliii, 1424.

mental evidence. The first question will be whether lecithin ever exists as an available compound in the activating serum and in the venom-susceptible corpuscles. To decide this point it is absolutely necessary to exclude other activators than lecithin from the activating serum or susceptible corpuscles. This having been done, we can compare the degree of firmness of lecithin-containing compounds of various kinds of serums and corpuscles, including the originally activating and non-activating serums and corpuscles. Even if we find that in one set of serums there is present an available lecithin compound, and not in the other, we shall then face a new problem, namely, whether the fact of availability or the looseness of the lecithin compound in the former serums is applicable to the whole blood or is to be attributed to a particular lecithin compound found only in the serum, but not in the blood corpuscles.

The part which lecithin may play in venom hæmolysis caused by fresh serum is not quite clear, inasmuch as serum contains still other complementary substances, which, doubtless, play their own rôles. A sharply differential method for venom hæmolysis caused by lecithin and by certain other complementary substances is required to clear up the question to what extent lecithin is responsible for the production of hæmolysis by venom in unmodified serums.

Before recording the results of my present investigations relating to the points already referred to above, as well as to some other questions to be introduced and considered later in this paper, a brief statement of the recent development of our knowledge concerning venom hæmolysis will make the necessity and object of the present work more obvious.

Snake venom produces hæmolysis only through the aid of a secondary substance. Thus Flexner and Noguchi<sup>4</sup> first demonstrated that the blood corpuscles of certain species of animals undergo venom hæmolysis when there is present suitable serum, and believed that complement of such serum is the activating principle. This was, however, found not to be always the case, as Kyes<sup>5</sup> discovered that there are instances where the corpuscles are dissolved without the addition of serum. The cause of the independent susceptibility of these corpuscles was ascribed to the presence of intracellular activator in these cells. The general characteristics of intracellular activators agreed with those of serum complement; Kyes called them endocomplements. At the same time lecithin

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<sup>4</sup> Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vi, 277.

<sup>5</sup> Kyes, *Berl. klin. Woch.*, 1902, xxxix, 886, 918.

was found to be capable of activating venom with striking readiness, but Kyes distinguished this form of venom hæmolysis from that caused by the complementary substances of certain fresh serums or by the endocomplement of certain susceptible corpuscles, by their resistance to high temperature and their activity at  $0^{\circ}\text{C}$ ., in which lecithin excelled the other. He also confirmed the important observation of Calmette<sup>9</sup> of the acquisition of power or the increase in strength of the venom activating property of various serums after heating to  $65^{\circ}\text{C}$ . and higher. This phenomenon was attributed by Keyes to the liberation of lecithin by the action of heat. While adding still further evidences to the occurrence of venom-complement hæmolysis, Keyes and Sachs<sup>10</sup> withdrew the opinion that endocomplement was a thermolabile complement, and presented a new view that it is really lecithin contained in the stroma of the susceptible corpuscles. The reason why the stroma of venom-resistant corpuscles does not react with venom was explained by the hypothesis that lecithin exists in an unavailable form in these corpuscles. In his third communication Kyes<sup>11</sup> succeeded in preparing a compound of venom and lecithin (lecithid) and threw suspicion on that mode of venom hæmolysis in which complement is thought to take a part.

Kyes suggested the possibility that any injurious substance may modify the corpuscles so as to render available the lecithin otherwise inaccessible to the venom. But this assumption has never been proven experimentally. My previous experiments<sup>8</sup> show lecithin to be by no means an inert compound, although its activity is likely to be underestimated on account of the slow reaction-time. Further, I was led to consider certain oleic compounds as well as oleic acid as venom activating agents.<sup>12</sup> This point has an important bearing on the present investigation of the venom activating substances of normal serum and susceptible corpuscles. Although no relationship between the lipolytic and the hæmolytic properties of venom and phytogenous toxalbumens have been established, yet the discovery by Neuberg and Rosenberg<sup>13</sup> of the lipolytic property of various venoms and bee poison is a fact of great interest. Ricin, which is a powerful lipolyzer,<sup>14</sup> forms a strong hæmolysin when mixed with free lecithin,<sup>15</sup> a fact important in that it shows that ricin does not unite with the lecithin in the integral corpuscles or serum. Apparently lecithin in the native condition in these substances is unattackable by ricin.

Hence it appears that we are again in the dark as to the real nature of venom hæmolysis. We are entirely unable to answer the question whether complement and certain complement-like bodies of serum have any part in venom hæmolysis, and whether lecithin

<sup>9</sup> Calmette, *Compt. rend. d. l'Acad. d. Sciences*, 1902, cxxxiv, 1446.

<sup>10</sup> Kyes and Sachs, *Berl. klin. Woch.*, 1903, xl, 21, 57, 82.

<sup>11</sup> Kyes, *Berl. klin. Woch.*, 1903, xl, 956, 982.

<sup>12</sup> Madsen and Noguchi, *Oversight over det Kongl. Danske Vedenskabernes Selskabs Forhandlungen*, 1904.

<sup>13</sup> Noguchi, *Jour. of Exper. Med.*, 1906, viii, 87.

<sup>14</sup> Neuberg and Rosenberg, *Berl. klin. Woch.*, 1907, xlv, 54.

<sup>15</sup> Pascucci, *Hofmeister's Beiträge*, 1906, vii, 457.

<sup>16</sup> Neuberg and Rosenberg, *loc. cit.*

is really present in available form for venom in activating serum and susceptible corpuscles. The sum of what we know at present is that blood serum and corpuscles yield large amounts of lecithin upon alcoholic extraction, that venom can form a powerful hæmolytic compound with free lecithin, that the activating property of serum and venom susceptibility of the corpuscles have no direct relation to their lecithin content, that certain activating serums contain venom activating principles which in some respects closely resemble complement, that certain chemicals, which may be present only in certain bloods as normal constituents, can produce a form of venom hæmolysis hardly to be distinguished from that caused by normal serum.

A ready way to clear up this confusing point is to discover an agent possessing the elective inhibitory action upon one or other of the venom activating principles. In course of my study<sup>14</sup> on the anticomplementary action of various acids, alkalies and salts, I found, among others, that various salts of the alkali earths inhibit complementary action without altering the serum amboceptors. Calcium chloride,<sup>15</sup> when used in a dilute solution, is most suitable to remove the complementary action of serum. This salt was employed in the present work to inactivate serum complement, as it has no marked destructive action upon venom amboceptor, when used in a strength of  $\frac{1}{10}$  N, or weaker. On the other hand, calcium chloride has no anti-activating power against lecithin. Venom lecithid retains its hæmolytic activity in a medium containing calcium chloride. When the amount of lecithin, or venom lecithid, is very small the salts retard complete hæmolysis, but have no ultimate effect on the process.

In addition to this differential agent an ethereal extraction of venom activators prepared from an active serum or the stroma of susceptible corpuscles was employed as a means to distinguish the lecithin-like activator from the complement-like ones. This method was especially useful for determining the protein-lecithid nature of the activator of certain serums and all heated serums. I shall return later to a discussion of this point.

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<sup>14</sup> Noguchi, read at the meeting of the American Society of Bacteriologists, New York, Dec. 28, 1906.

<sup>15</sup> 0.85 per cent. of this salt is isotonic.

## VENOM ACTIVATORS OF FRESH SERUM.

To determine whether the venom activating property of fresh serum is removed by calcium chloride, 0.5 c.c. of the serum under consideration was mixed with 0.5 c.c. of  $\frac{1}{10}$  N. solution of this salt, and the volume made 2 c.c. with 0.9 per cent. salt solution. The washed corpuscles were added in proportion of five per cent. After an incubation of half an hour at  $37^{\circ}$  C., 0.1 c.c. of 0.4 per cent. solution of cobra venom was introduced, and the whole incubated for several hours at  $37^{\circ}$  C. and left at room temperature for the rest of the night. The result was read off, as a rule, within twenty-four hours. The corpuscles and serum came from the same blood; but sometimes the washed corpuscles of a second blood were employed. This last combination is, of course, possible only when the serum in question has no hæmolytic action upon the corpuscles. Table I. shows the results obtained with the serum and corpuscles of the same blood.

TABLE I.

Blood of	Control.	CaCl <sub>2</sub> Addition.
	Fresh Serum 0.5 c.c. } + Corpuscles. } + 0.9 % NaCl 1.5 c.c. }	Fresh Serum 0.5 c.c. } + Corpuscles. } + N/10 CaCl <sub>2</sub> 0.5 c.c. } + 0.9 % NaCl 1.0 c.c. }
	0.1 c.c. of 0.1 % Cobra Venom Solution.	0.1 c.c. of 0.1 % Cobra Venom Solution.
Man	complete hæmolysis	no hæmolysis
Horse	"	"
Pig	"	"
Cat	"	"
Guinea-pig	"	"
Rabbit	"	"
Pigeon	"	"
Hen	"	"
Goose	"	"
Goat	no hæmolysis	"
Ox	"	"

The above experiment shows that the majority of the varieties of blood employed undergo complete hæmolysis by venom and that their activating substances are completely inhibited by calcium chloride. The absence of hæmolysis in the presence of CaCl<sub>2</sub> is not due to destruction of the venom, because the addition of a small quantity of free lecithin to such mixture produces complete hæmolysis. Or, the presence of a small amount of lecithin, which may be added purposely at the same time as the CaCl<sub>2</sub>, leads to complete hæmolysis. There is still another way to prove that the corpuscles are sensitized by venom in the CaCl<sub>2</sub> mixture. The corpuscles may



be washed with 0.9 per cent. salt solution and finally suspended in a fresh lot of the latter. If now a small amount of lecithin or 0.5 c.c. of any activating serum is added complete hæmolysis occurs.

Whether or not the venom activators of the serums are identical with compounds contained in them cannot be determined from this experiment, as both are inactivated by  $\text{CaCl}_2$ . At all events, lecithin is excluded by this test as the activating agent of these serums.

The serum of the dog is extremely rich in venom activating substances and differs from other serums in its relation to inactivation with calcium chloride. Of twelve different samples of normal dog serum complete inactivation by  $\text{CaCl}_2$  was obtained only in three, marked delay, in five, and slight retardation, in the rest. In this series homologous and heterogeneous corpuscles were tested and gave the same result. Thus dog's serum is an example of a fresh serum, in which lecithin exists in an available form for venom activation. The simultaneous presence of certain  $\text{CaCl}_2$  inhibiting venom activators was also demonstrable; and in a few instances this class of activators alone was present in the dog serum.

#### VENOM ACTIVATORS OF BLOOD CORPUSCLES.

A wider variation is exhibited by the corpuscles of different species of animals in their action toward venom. Some kinds of corpuscles are promptly, while other not at all dissolved by venom. The latter class of corpuscles remains undissolved because of the absence of suitable venom activators although sensitization still occurs. The corpuscles of all bloods undergo hæmolysis when suitable activators are present. The most susceptible corpuscles are those of dog and guinea-pig, and entirely refractory are those of ox, goat and sheep. The corpuscles of horse, rabbit, rat, pig and man occupy an intermediary position. Those which approach the limit of the series of non-susceptibility require a longer incubation for hæmolysis than those which stand near the opposite end. This incubation time can be greatly shortened by adding certain activating serums. The original assertion of Flexner and Noguchi<sup>19</sup> that venom requires complement for its activation was based on the fact that certain washed corpuscles remained intact

<sup>19</sup> Flexner and Noguchi, *Jour. of Exper. Med.*, 1902, vii, 277.

during a period of experimentation in which the same corpuscles underwent complete hæmolysis when fresh serum was added. In a later paper Flexner and Noguchi<sup>17</sup> described the presence of complementary substances in various kinds of organic cells.

The cause of destruction of certain corpuscles by venom alone is ascribed by Kyes<sup>18</sup> and Kyes and Sachs<sup>19</sup> to the presence of endocomplement. The thermolabile nature and the inactivity at 0° C. of endocomplement led them first to classify it with complements. But later they showed that the thermolability is caused by the simultaneous presence of hæmoglobin and hence concluded that it was the lecithin of the stroma. To this particular point I will return a little later.

In my experiment regarding the nature of intracellular venom activators of the integral corpuscles, I employed calcium chloride to determine whether this salt can suppress hæmolytic action of venom upon susceptible corpuscles. The result was rather remarkable. The washed corpuscles of horse, rat, rabbit, cat, guinea-pig, pig, pigeon, goose, hen and man remained undissolved in  $\frac{1}{10}$  N. to  $\frac{1}{100}$  N.  $\text{CaCl}_2$  medium in the presence of cobra venom. The controls dissolved completely in from fifteen minutes to twenty-four hours. The protection of the susceptible corpuscles is not due to the destruction of venom by  $\text{CaCl}_2$ , because by a later addition as well as by the simultaneous introduction of a small amount of lecithin, or even by the addition of old dog serum (inactive with age) complete hæmolysis can be induced. The least susceptible corpuscles require the smallest amount of  $\text{CaCl}_2$  for protection. On the other hand, a trace of hæmolysis is often observed with the corpuscles of guinea-pig or man after twenty-four hours. Different samples of dog corpuscles behave differently. I employed several samples and with some there is perfect protection, with others partial protection. Dog corpuscles are liable to spontaneous hæmolysis within twenty-four hours, which fact may be responsible for irregular results.

The removal of intracellular activators by means of digestion

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<sup>17</sup> Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.

<sup>18</sup> Kyes, *loc. cit.*

<sup>19</sup> Kyes and Sachs, *loc. cit.*

in a calcium chloride solution gave variable results. The corpuscles of horse or pig are protected from venom after digestion in  $\frac{1}{80}$  N.  $\text{CaCl}_2$  for half an hour, but those of guinea-pig and dog are not. If we use  $\frac{1}{10}$  N. solution these corpuscles are rendered even more susceptible to venom than the undigested samples. The mechanical injuries brought about during the procedure of washing away the calcium salt with sodium chloride solution may have some influence. Perhaps a sudden change of tonicity and breaking up of agglutinated masses of corpuscles hasten the destruction. Hence it would appear that in the majority of the corpuscles lecithin seems not to be able to play a part as activator. Before deciding this matter finally other considerations should be taken up. The presence of hæmoglobin in lecithin containing fluid, or the addition of lecithin to the corpuscular solution does not permit of the inactivation by calcium chloride. Ovovitellin of hen's egg contains a high percentage of lecithin. This lecithin-proteid is not split up with ether, but by hot alcohol. I prepared a saline suspension of ovovitellin and examined it for venom activation. It was found to be an excellent activator. Hence the question arose whether this proteid compound could not be easily inactivated by calcium chloride. This proved not to be possible. Thus lecithin, either in a mechanical mixture with hæmoglobin or corpuscular contents, or in a chemical combination with proteid, is not inactivated by  $\text{CaCl}_2$ . That lecithin does not exist in an available form for venom hæmolysis in susceptible as well as insusceptible corpuscles can be shown by the following experiments.

The washed corpuscles of the guinea-pig were broken up with about three times their volume of water. The stroma was separated out by adding 0.9 per cent. sodium chloride and the precipitated stroma was collected by centrifugation. The clear, intensely dark-red supernatant fluid was pipetted off and used for some tests, while the stroma was twice washed in a 0.9 per cent. sodium chloride solution.

The pink-colored stroma was tested for its venom activating property, for which the corpuscles of ox and goat were used. The activation was not rapid, but progressed rather slowly. The deep-red supernatant fluid was not activating. The test with calcium chloride showed that the activating property of the stroma is completely removed by this salt. The shaking of the stroma with a large quantity of ether also removed this property. On the other hand, the ethereal extract upon evaporation left a small amount of fatty substances, which contained chiefly aceton-soluble fats, but almost no lecithin to be detected even with venom.

I redissolved the oily mass in 0.9 per cent. salt solution, in which it showed turbidity and an acid reaction to litmus. This suspension is activating.

We have thus succeeded in locating the site and identifying the probable nature of the phosphorus-free fat acting as an intracellular venom activator. It may be added that the activating property of this suspension is completely set aside by calcium chloride. As Kyes and Sachs first showed, lecithin could be isolated from the stroma with hot alcohol. The lecithin obtained from it is activating, but entirely different from the activators of the integral corpuscles, corpuscular solution and ethereal extract of corpuscles in its relation to  $\text{CaCl}_2$ . While lecithin can be extracted with alcohol from any of the corpuscles, no matter whether they belong to the susceptible or the insusceptible class, in nearly equal quantities, the ether-soluble venom activators are found only in the corpuscles susceptible to venom. I have tried many times to extract an ether-soluble venom activator from the corpuscles of the ox and goat, but without success. These corpuscles are naturally venom-resistant, and their corpuscular solutions do not contain a venom activator. A later experiment with the corpuscles of the dog gave practically the same result as those of guinea-pig.

Direct evidence that certain fatty acids, soaps and neutral fats are capable of acting as venom activators can easily be given. The venom-resistant corpuscles are washed and freed from the serum. Then the minimal hæmolytic quantities of oleinic acid, sodium oleate, ammonium oleate, neurin oleate and triolein are ascertained. The subminimal hæmolytic dose of any of these chemicals is taken and mixed with the corpuscular suspension of any of the insusceptible bloods. No hæmolysis occurs after twenty-four hours. But if an adequate quantity of cobra venom is added at the same time, complete hæmolysis occurs. The addition of these chemicals to the corpuscular solution (not suspension) of an insusceptible blood renders it venom-activating. This artificially prepared, activating solution of blood corpuscles behaves in the manner of the solution of susceptible corpuscles, and becomes inactive upon ethereal extraction in case of oleinic acid, organic soaps, and triolein. With the solutions whose activating property is conferred by the addition of alkaline oleate soaps, ether fails to remove it. Calcium

chloride is very effective in depriving these solutions of their acquired venom-activating property. The result with triolein is not satisfactory, as two preparations from Merck and Kahlbaum gave entirely different results, as the latter was almost entirely inactive. The activators of susceptible corpuscles were completely and permanently inactivated by boiling.

From these results it seems justifiable to conclude that lecithin does not exist in the corpuscles, irrespective of their susceptibility to venom hæmolysis, in an available form for venom. The degree of susceptibility of blood corpuscles depends chiefly upon the amount of ether-soluble activators contained in the cells. These ether-soluble activators are, doubtless, fatty acids, and especially oléinic acid. The absence of fatty acids in the insusceptible corpuscles is in perfect harmony with the reaction to venom. If we compare the amount of fatty acids<sup>20</sup> and the degree of venom susceptibility of different corpuscles we discover that a definite and undeniable parallelism between these two factors exists. If any amount of venom activators are present in the stroma after ethereal extraction it does not necessarily follow that they are lecithin, because the ether-insoluble soaps may be preëxistent or be formed during the manipulation of the corpuscles with water, and they would certainly take on a good share in the activation.

#### VENOM ACTIVATORS OF HEATED SERUM.

Calmette discovered that any serum acquires venom activating property or has its property increased by heat above 65° C. Kyes found that the maximum effect is reached at 100° C. and coincides with the maximum liberation of lecithin. That the activating property of such heated sera is due to lecithin can be further established by means of chloride of calcium, as the next table shows.

An attempt was made to ascertain whether lecithin exists in a free state or as a proteid compound in heated serum. That a non-coagulable proteid exists in serum heated to 80° to 100° C. in a neutral or slightly acidified reaction has long been known. Chabrière,<sup>21</sup> who first described this proteid, called it albumon. Howell's<sup>22</sup>

<sup>20</sup> Abderhalden, *Zeit. f. Physiol. Chem.*, 1898, xxv, 65.

<sup>21</sup> Chabrière, *Compt. rend. de l'Acad. de Sciences*, 1891, cxiii, 557.

<sup>22</sup> Howell, *Amer. Jour. of Physiol.*, 1906-7, xvii, 280.

TABLE II.

	Serum 0.2 c.c. Corpuscles 5 per cent. Cobra Venom 0.1 per cent., Solution 0.1 c.c. (added later).						{ Total Volume made to 2 c.c. with 0.9 per cent. NaCl Solution.			
	Fresh.		56° C.		65° C.		85° C.		100° C.	
	Saline.	CaCl <sub>2</sub> N/10 0.5	Saline.	CaCl <sub>2</sub> N/10 0.5	Saline.	CaCl <sub>2</sub> N/10 0.5	Saline.	CaCl <sub>2</sub> N/10 0.5	Saline.	CaCl <sub>2</sub> N/10 0.5
Man	C. H.	None	Slight H.	None	Much H.	Moderate	C. H.	C. H.	C. H.	C. H.
Horse	"	"	"	"	"	Much H.	"	"	"	"
Guinea pig	"	"	C. H.*	"	C. H.	C. H.	"	"	"	"
Ox	None	"	None	"	Slight H.	Slight H.	"	"	"	"
Goat	"	"	"	"	"	"	"	"	"	"

recent investigations of this proteid added many new facts. Albumon is characterized as soluble in water, indiffusible through celloidum membrane, non-coagulable in presence of neutral or acid reaction upon boiling, but easily precipitable by half saturation with ammonium sulphate. It contains a large percentage of phosphorus and iron. The phosphorus represents the lecithin which exists in its molecule as a component extractable only by warm alcohol. Howell considers albumon an artificial product formed through the action of the heat, while some previous investigators considered it a normal constituent, or were uncertain on this point.

In my experiments I employed ox serum. The serum was diluted with three times its volume of water and gradually boiled at neutral reaction (neutralization by means of acetic acid). The non-coagulable portion was separated from the coagulum by filtration. The clear fluid gave the required characteristics for albumon, and upon examination it proved to be highly active for venom and not to be inactivated by calcium chloride. Ether failed to extract any activator from the fluid medium or after drying. On the other hand, warm alcohol yielded much lecithin and perhaps some other venom activating substances. The proteid precipitated out by the process of extraction was inactive. Goat serum yielded a similar albumon.

Thus the conclusion seems warranted that the lecithin of heated serum does not exist in a free state, but in a proteid compound, which is capable of reacting with venom. In this case the pre-existence of this proteid in unheated serum is conclusively excluded by the fact that neither the fresh serum, nor the coagulum of these bloods contained any available lecithin compounds.

\* Guinea-pig corpuscles contain endocomplement, which accounts for complete hæmolysis. If ox or goat corpuscles are used no hæmolysis results, while the same corpuscles are readily dissolved by the addition of over-heated serum, and cannot be reduced by CaCl<sub>2</sub>.

LOCATION OF AVAILABLE LECITHIN IN VARIOUS PROTEID FRACTIONS  
OF SERUM.

The existence of venom activators of lecithin nature in normal dog serum has been mentioned. But the question whether the availability of lecithin in this particular serum is due to looseness of a lecithin-proteid compound, or to the presence of a definite lecithin compound peculiar to this serum was left undecided.

Two different samples of dog serum were dialyzed in collodium sacs against running water and after seventy-two hours the whitish precipitates were collected by filtration and dissolved in 0.9 per cent. sodium chloride solution. The filtrate was made isotonic with the same salt. The first represents the serum globulin and the second the serum albumin. These were now tested for venom activating property. The result shows that the globulin fraction was inactive and the albumin highly active. The same serums were then fractionated with ammonium sulphate. The precipitate produced by half saturation was separated from the serum by filtration. The filtrate was then completely saturated by further addition of this salt. The precipitate was obtained by filtration. The first represents the globulin and the second the albumin fraction of the serum. Both were dialyzed in collodium sacs for seventy-two hours. In the first sac, which contained the globulin fraction, there was a large amount of whitish precipitate. This was filtered and collected; the filtrate was also preserved for testing. The content of the second sac was perfectly clear, and was used for the tests. All three were made isotonic with sodium chloride. The globulin precipitate dissolved in this medium with slight opalescence. The tests for venom activation gave the following result: the solution of the globulin precipitate and the albumin fraction were unable to activate venom, while the clear portion of the first sac was extremely active. This last fraction was non-coagulable in saline solution upon boiling, but precipitable by ammonium sulphate and heat and coagulable by alcohol. It gave a blue-violet Biuret reaction. The venom activating property of this portion was unaffected by  $\text{CaCl}_2$  or ethereal extraction.

Similar tests with ox and pig serums (both non-activating) gave results differing from those with dog serum, as the globulin lacked the venom activating property.

The next question was whether the globulin and albumin fractions of these serums contain any lecithin. The examination was made by extracting the serums with hot alcohol ( $70^\circ \text{C}.$ ).

The globulin fraction of the serums of dog, horse, ox and pig yielded upon hot alcoholic extraction comparatively small amounts of lecithin. On the other hand, the albumin fraction of these serums yielded much more lecithin than the globulin. The non-coagulable portion obtained from the first sac (containing the pre-

precipitates by half saturation with ammonium sulphate) contained a large amount of lecithin.

The globulin as well as albumin fractions did not produce upon boiling, either with or without sodium chloride, as much lecithin (in available form for venom) as the whole serums from which they were isolated. In some instances even considerable reductions were observed with the globulin fraction plus the non-coagulable lecithin proteid of dog serum. I am inclined to ascribe this reduction in activity to the simultaneous presence of cholesterol in the medium. The problem, which was set at the beginning of this topic, has been resolved experimentally: the venom activating substance of lecithin nature in dog serum exists as a definite proteid compound, and does not depend upon a loose combination between lecithin and globulin or albumin. The non-venom-activating serums do not contain a similar lecithin proteid to that found in dog serum.

#### PREPARATION OF ARTIFICIAL VENOM ACTIVATING SERUM.

Normal serum may contain two different sets of venom activators, namely, one resembling complement and the other a proteid compound of lecithin resembling albumin. The latter is occasionally present in normal dog serum, while the former constitute the venom activating substances contained in all venom activating serums. Calcium chloride renders the first class of activators ineffective. The venom activating property of these serums, which are subject to the calcium inactivation, is completely or in part removable by ethereal extraction. The ethereal extract contains fatty acids and neutral fats; but not lecithin. The inactivation of these serums by the temperature of  $56^{\circ}$  C. is rather uncertain, although more or less reduction in activity is observed. Sometimes complete loss is obtained. The activating function of this class of venom activators is suspended at  $0^{\circ}$  C. Except in certain herbivorous mammalian bloods (ox, goat and sheep) the majority of mammalian serums and certain avian serums contain the complement-like venom activators in varying quantities, and no lecithin-like activators are to be found in these serums. Even in dog serum, which contains an available compound of lecithin, there is



a large amount of the other present. I have already mentioned that in the ethereal extract of these serums varying quantities of fatty acids and fats are contained. But more convincing is the fact that none of the non-activating serums yields more than a minute quantity of activating fatty acids.

By the term "venom activating fatty acids" oleic acid is chiefly meant. Palmitic and stearic acid are far less effective in this respect. The more oleic acid is present in the ethereal extract, the stronger is the venom activating property.

Apart from the varieties of the fatty acids, the whole amount of these acids extractable from dog serum is nearly twice as much as from the serum of ox, goat or sheep.<sup>23</sup> I have already mentioned that two samples of triolein which I used acted very differently, so that I have been put in doubt regarding my earlier experiments.<sup>24</sup>

The facts above enumerated clearly indicate that fatty acids have a direct relation to the venom activating property of blood serum.

Leaving aside the question whether fatty acids represent the entire venom activators or only part of them, I will consider next whether or not certain fatty acids and soaps can confer the venom activating property upon non-activating serums.

For this experiment two sets of normal ox serum were used: the one without any modification, and the other after being shaken with a large volume of ether. To both sets oleinic acid in amounts of 0.12<sup>25</sup> to 100 grams of the serum was added. In still other series, and with ox serum, sodium oleate was used instead of oleinic acid. Oleinic acid and sodium oleate are highly hæmolytic if these are dissolved in this concentration (or emulsified in case of oleinic acid) in a 0.9 per cent. sodium chloride solution; but in ox serum they remain completely inactive. When these mixtures are used as venom activators they display very powerful hæmolytic action upon insusceptible corpuscles (ox and goat). Their action is not so prompt as that of dog serum or any heated serum, but more like that of guinea-pig serum, except that it is prompter than that. By

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<sup>23</sup> Abderhalden, *loc. cit.*

<sup>24</sup> Noguchi, *Jour. of Exper. Med.*, 1906, viii, 87.

<sup>25</sup> This proportion was taken from the data given by Abderhalden for dog serum.

reducing the quantity of the mixture a descending scale of activation can be secured until no effect whatever is obtained. There is no marked difference between the acid and soap in the mode of action. Calcium chloride completely inactivates these mixtures. The temperature of  $56^{\circ}$  C. has a marked reducing power upon the soap and serum mixture, but hardly any upon the acid and serum mixture. Ethereal extraction removes the activating property of the latter, but not of the former. At  $0^{\circ}$  C. the latter is still slightly active, but not the former.<sup>26</sup>

These artificially prepared venom activating serums are not easily distinguishable from certain natural serums.

The second type of venom activating serum, in which lecithin is present in an available form, can be artificially prepared, by adding pure lecithin, or the non-coagulable proteid of heated serum, to non-activating serum. When the mixture is made its activating property cannot be stopped by calcium chloride or the temperature of  $0^{\circ}$  C. Cholesterin inhibits the effect of this mixture. Ether cannot remove much of the lecithin when once mixed with the serum; this perhaps may be due to the fact that lecithin can enter into combination with some of the serum components.<sup>27</sup>

#### THE PROTECTIVE ACTION OF CALCIUM CHLORIDE AGAINST VENOM CYTOLYSIS.

The powerful cytolytic property of various kinds of venoms upon the cells of liver, kidney, nerve, testis, and ova of different animals has been demonstrated by Flexner and Noguchi.<sup>28</sup> The mechanism of the cytolysis was found to be essentially the same as that of hæmolysis and that intracellular complements played an important part. Knowing no other means to eliminate the intracellular complement at that time, Flexner and Noguchi employed heat (temperature of  $55^{\circ}$  C. maintained for thirty minutes). They found that this temperature rendered the cells insusceptible to venom, unless fresh serum was added to the mixture. In this way the similarity of venom cytolysis and serum cytolysis was established.

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<sup>26</sup> No suppression of action without serum constituents.

<sup>27</sup> Mayer and Terroine, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 398.

<sup>28</sup> Flexner and Noguchi, *Jour. of Path. and Bact.*, 1905, x, 111.

Since I found in calcium chloride a powerful anti-complementary hæmolytic substance it was natural to expose the somatic cells to the influence of this chemical to establish the nature of the complementary body present in them. In this series of experiments the cells of liver, kidney, testis and brain of the guinea-pig and rat were employed. It was difficult to obtain many ganglion cells in good condition, while the testicular cells were easily obtained intact. Uniform emulsions (5 per cent.) in salt solution of these cells were measured into small test-tubes to which daboia venom was added in proportion of 1 : 100. The mixture was placed in a water thermostat at 37° C. for five hours and examined microscopically. To test the action of calcium chloride the salt was added to the suspensions of these cells in ratio of  $\frac{1}{10}$  N., and half an hour later the venom was introduced.

The results are briefly as follows: the cells of the liver, kidney and testis are well preserved in the saline solution after five hours. The ganglion cells are less distinct in outline, but apparently have not disintegrated (controls). In the venom solution the testicular cells are nearly all dissolved, but the spermatozoa minus heads remain. The clearing up of the cellular elements is distinctly visible *in vitro*. The liver cells become more or less swollen and the outlines indistinct. The granules disappear and the nuclei become more distinct. Agglutination of free cells is marked. The kidney cells and tubules become gradually indistinct and a general disintegration of the former occurs. The number of cells is less than in the controls; agglutination occurs. The ganglion cells have disappeared wholly, and a general solution of granular elements has taken place. These alterations are much more pronounced in the emulsions of guinea-pigs' than of rats' brains. Not only the microscopical, but also the macroscopical appearances of the venomized emulsions is at once recognized by a marked clearing up of the original turbid suspensions. The addition of calcium chloride completely prevents the destructive action of venom upon the cells. The protection afforded by this salt is greater than is obtained by heating the cells to 55° C.

From these facts the complement-like nature of the venom activators contained in the somatic cells is once more established.

## SUMMARY.

In normal serums of the majority of mammalian and avian blood there exists certain substances capable of activating venom hæmolyisin. They are extractable from serum by means of ether, and are capable of conferring upon the originally non-activating serum a power to activate venom, when mixed with the latter. The ethereal extract consists of fatty acids, neutral fats and possibly also some ether soluble organic soaps. The fatty acids and soaps, especially of the oleinic series, acquire certain characteristics of complements in general, when they are mixed with serum. They are inactive without the venom in the mixture; they are inactivable with calcium chloride; they exhibit a tendency to go off in activity with age; they are inactive or only weakly active at 0° C., and they are extractable by ether. In testing the serum from which the ether soluble substances are removed, it is found that no venom activating property is left. Warm alcoholic extraction of such serum yields, however, a large quantity of lecithin. In the case of non-activating serums no venom activating fats appear in the ethereal extract. Lecithin exists in such serum in no less quantity than in the activating kind.

The addition of oleinic acid or its soluble soaps to a non-activating serum, in a ratio which corresponds to the percentage of fatty acids or soaps contained in some of the easily activating serums, will make the serum highly active in regard to venom.

In normal serum of dog there exists, besides the group of activators already mentioned, another kind of venom activators which has been identified as a lecithin compound acting in the manner of free lecithin.

A very sharp differentiation of the hæmolysis produced by this activator and by the other groups of activators is obtained by means of calcium chloride, which is powerless against lecithin or lecithin compounds, but effective in removing the action of the latter. This lecithin containing proteid can be precipitated by half saturation with ammonium sulphate, but is perfectly soluble in water, and is not coagulated in neutral alkaline salt solutions upon boiling. Alcohol precipitates a proteid-like coagulum and extracts lecithin from it; ether does not extract lecithin from this compound.

Non-activating serums do not contain any such lecithin compound.

Lecithin contained in other serum proteids, mainly as lecithalbumin, and perhaps as contained in globulin, is not able to activate venom. This is true of all the serums with which I worked; it matters not whether these fractions (obtained with ammonium sulphate) belong to the most activating serum (dog) or to the non-activating serum (ox).

The non-coagulable portion of all heated serum contains a venom activator of the nature of lecithin. This activator is contained in a non-coagulable proteid described by Howell which is identical with Chabrié's albumon. As there is no ether-extractable lecithin in this portion of the serum, the activating property of heated serum must be due to this proteid compound of lecithin. That this lecithin proteid does not pre-exist in normal serum but is produced by the action of high temperature is true of all serums except that of the dog. In venom activation we know now that lecithin becomes reactive with venom when it is transformed from other proteid compounds into the non-coagulable form, the albumon. Howell's view of the non-existence of the non-coagulable proteid in normal serum seems to receive a biological support from venom hæmolysis.

Ovovitellin derived from hen's egg is one of the best venom activators of the lecithin proteid type.

The cause of venom susceptibility of various kinds of blood corpuscles does not depend upon the existence of lecithin in the corpuscles, but solely upon the amount of fatty acids, and perhaps, also, soaps and fats, contained in the corpuscles. The protection which calcium chloride gives against venom hæmolysis is proof of the absence of lecithin activation. From the stroma of susceptible corpuscles fatty acids or some fats can be extracted with ether. After ethereal extraction the stroma becomes non-activating, while the extract contains fatty acids and some soaps or fats, which when added to venom-resistant corpuscles render the latter vulnerable to venom. The corpuscular solution of non-activating corpuscles does not contain enough fatty acids. The larger the amount of fatty acids and soaps in the corpuscles, the easier the cells undergo

venom hæmolysis. Lecithin exists in the stroma of all kinds of corpuscles, but in a form unavailable for venom activation.

The somatic cytolytic processes caused by venom requires intracellular complements. The experiments performed on the cells of liver, kidney, testis and brain of the guinea-pig and rat indicate that the substances which act as complements are inactivable by calcium chloride.

## ON THE INFLUENCE OF THE REACTION AND OF DESICCATION UPON OPSONINS.<sup>1</sup>

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Whether the opsonic property of normal and immune serums, to which the writings of Wright and Douglas<sup>2</sup> have called renewed attention and endowed with special significance, is a new and hitherto undescribed property of serum or a special function of the usual immune bodies of serum is still a question of dispute.

Bulloch and Atkin,<sup>3</sup> Neufeld and Rimpau,<sup>4</sup> Barratt,<sup>5</sup> Neufeld and Töpfer,<sup>6</sup> Hektoen,<sup>7</sup> and Keith<sup>8</sup> share the view of Wright and Douglas that the opsonic action is due to the presence of certain hitherto unrecognized, distinct bodies, while Savtchenko,<sup>9</sup> Besredka,<sup>10</sup> and Dean<sup>11</sup> are inclined to consider the opsonins as identical with the amboceptors (fixators). Quite recently Muir and Martin<sup>12</sup> brought forth evidence that opsonization depends upon the coöperation of two substances, one of which, at least, closely resembles complement of serum in certain of its biological properties. Still later Levaditi and Inmann<sup>13</sup> asserted that opsonin are nothing but the complements of serum.

It is not my intention to enter into this discussion, but, for my purpose, it is necessary to mention that in common with complement, so-called, the opsonins are absorbed or fixed by sensitized bacteria, blood corpuscles, specific precipitates,<sup>14</sup> and indifferent

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<sup>1</sup> Received for publication April 30, 1907.

<sup>2</sup> Wright and Douglas, *Roy. Soc. Proc.*, 1903, lxxii, 357; 1904, lxxiii, 128.

<sup>3</sup> Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.

<sup>4</sup> Neufeld and Rimpau, *Deut. med. Woch.*, 1904, xxx, 1458.

<sup>5</sup> Barratt, *Roy. Soc. Proc.*, 1905, lxxvi, 524.

<sup>6</sup> Neufeld and Töpfer, *Centralbl. f. Bakt.*, etc., 1905, xxxviii, 456.

<sup>7</sup> Hektoen, *Jour. of Infect. Diseases*, 1906, iii, 434.

<sup>8</sup> Keith, *Roy. Soc. Proc.*, 1906, lxxvii, 573.

<sup>9</sup> Savtchenko, *Ann. d. l'Inst. Pasteur*, 1902, xvi, 106.

<sup>10</sup> Besredka, *Ann. d. l'Inst. Pasteur*, 1904, xviii, 363.

<sup>11</sup> Dean, *Roy. Soc. Proc.*, 1905, lxxvi, 506.

<sup>12</sup> Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

<sup>13</sup> Levaditi and Inmann, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 725.

<sup>14</sup> Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

bodies<sup>15</sup> as well as non-sensitized bacteria,<sup>16</sup> and exhibits thermolability and susceptibility to deterioration by age, similar to that which complement exhibits. Besides the liability to undergo adsorption and the high degree of thermolability, no other characteristics of ferments have been ascribed to opsonins. Were opsonins recognized as special ferments certain facts of theoretical and practical import should follow. Among other things we should look for an optimum of activity to be exhibited in the presence of a certain reaction which might or might not be that of the sample of serum which is being studied. If the degree of activity depended upon a given reaction then to obtain the optimum indication, upon which alone a measure of the quantity of opsonin could rest, this reaction would have to be secured. I have, therefore, made a study of the influence of the reaction upon the opsonic power of several blood serums for *B. typhosus*, *B. dysenteriae*, *Streptococcus*, and *Staphylococcus aureus*.

The results of these experiments harmonize so well that those alone relating to *Staphylococcus aureus* will be given in this paper.

#### THE INFLUENCE OF THE REACTION UPON OPSONINS.

*The Influence of Alkalinity.*—The degree of alkalinity of several serums were first titrated. Lacmoid paper was employed as indicator. The titration was conducted in the following manner. To 1 c.c. of serum <sup>10</sup>N. solution of hydrochloric acid was gradually added until the reaction reached neutral. The amount of acid required for complete neutralization was taken as representing the degree of alkalinity of each serum. The resulting fluid was then made up to 5 c.c. with 0.9 per cent. salt solution. Thus the final mixture had a strength of one fifth of the original serum. By this means it was possible to prepare a series of mixtures, in which 0.5, 0.3, 0.2, 0.1 and null alkalinity were left unneutralized. These fluid mixtures were used for opsonization, and the results are given in Table I. The cross signs under each column represent the average number of the bacteria taken up by a phagocyte, each cross standing for three bacteria.

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<sup>15</sup> Simon, Lamar and Bispham, *Jour. of Exper. Med.*, 1906, viii, 651.

<sup>16</sup> Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.



The bacteria were *Staphylococcus aureus*, twenty-four hours old, and the leucocytes human. The tubes were incubated at 37° C. for thirty minutes, and the technique employed was that given by Wright.

TABLE I.

	Varying Quantities of $\frac{1}{10}$ N. HCl Added to Leave the Indicated Degree of Alkalinity Unneutralized. Total Volume 5 c.c.					
	Original Alkalinity.	Alkalinity Left 0.5 c.c.	Alkalinity Left 0.3 c.c.	Alkalinity Left 0.2 c.c.	Alkalinity Left 0.1 c.c.	Neutral Reaction.
Dog	0.5 c.c.					
Ox	+++ 0.75	+++	+++	++++	++++	++++
Pig	++++ 0.8	++++	++++	+++++	+++++	+++++
Rabbit	+++++ 0.65	+++++	+++++	+++++	+++++	+++++
	++++	+++	+++	++++	++++	++++

As the foregoing table shows, a greater degree of opsonization is obtained at the neutral reaction than at the inherent alkaline reactions of the serums.

For the next series of experiments the inhibitory action of alkalinity upon opsonins was made more apparent by increasing the alkali in an ascending scale.

TABLE II.

	Original Alkalinity.	Amount of $\frac{1}{10}$ N. NaOH Added. Total Volume 5 c.c.				
		0	0.5 c.c.	1 c.c.	2 c.c.	$\frac{1}{3}$ c.c.
Dog	0.5 c.c.	+++	++	+	Negative	Negative
Ox	0.75	++++	+++	+	"	"
Pig	0.8	+++++	+++	+	"	"
Rabbit	0.65	++++	++	+	"	"

When 2 c.c. of  $\frac{1}{20}$  N. sodium hydrate solution are added to 1 c.c. of a serum the opsonizing property of the latter completely dis-

TABLE III.

Neutralized Serum.	Amount of $\frac{1}{10}$ N. NaOH Added. Total Volume 5 c.c.					
	0	1.2 c.c.	1.4 c.c.	1.6 c.c.	1.7 c.c.	1.8 c.c.
Dog	++++	++	+	±	Negative	Negative
Ox	+++++	++	+	±	"	"
Pig	+++++	+++	+	±	"	"
Rabbit	++++	++	+	±	"	"

appears; 1 c.c. of this solution added to 1 c.c. of serum reduces the opsonic activity to the minimum. Table III shows the maximum alkalinity in which opsonins are still able to act.

An alkali content approaching 1.6 c.c. suppresses the activity of opsonins. It is rather remarkable that this degree of alkalinity is only about twice as high as that possessed by the majority of normal serums.

In Table I. it is shown that the activity of opsonin is greater at the neutral point than at the alkalinity possessed by normal serums, but the difference was not a marked one. In the following experiment the inhibitory influence of the native alkalinity is brought to light by means of dilution. The normal serum of the pig was neutralized with hydrochloric acid and divided into two portions. To one portion sodium hydrate solution was added to make it contain just enough alkali to reproduce the original degree of alkalinity, namely, 0.8 c.c. of  $\frac{1}{10}$  N. To the second portion no alkali was added, but it was employed in the neutral reaction. These two portions were used for opsonization in ascending dilutions.

TABLE IV.

Dilution of the Neutralized Serum (Pig).	Alkalinity $\frac{1}{10}$ N. NaOH 0.8 c.c.	Alkalinity $\frac{1}{10}$ N. NaOH 0.8 c.c. Neutralized Back with $\frac{1}{10}$ N. HCl 0.8 c.c.	Control in Saline with Neutralized Serum Alone.
1:5	+++++	+++++	+++++
1:7	++++	++++	++++
1:10	+++	+++	+++
1:15	+	+++	+++
1:20	±	++	++
1:25	—	++	++
1:30	—	++	++
1:35	—	+	+
1:40	—	+	+
1:50	—	—	—
1:60	—	—	—
1:80	—	—	—

As Table IV. shows, the alkali restrained the opsonic action markedly and the minimum opsonization with the alkalized portion was between 1:15 and 1:20, while the neutral portion was still active at 1:30 to 1:40 dilutions. This restraining effect of alkali was not so evident in the concentrated state of the serum, but was rapidly developed as the dilution was increased. It may

be remarked in passing that the inhibitory influence of the reaction on ferments is by no means a quantitative one. No matter how large a quantity of ferment is present in a mixture no action follows if the reaction is highly unfavorable. When the reaction is merely such as to inhibit partially, only so much effect is obtained as the degree of optimum permits. Under such circumstances, the greater portion of ferment may remain inactive. It is only when ferment finds itself in a fluid of optimum reaction that real quantitative differences can be manifested. The opsonic indices can seldom be driven beyond four, and usually not beyond two or three, in spite of repeated vaccination with bacteria. The reason for this limit is not at once apparent, but it is not improbable that even here the reaction of the serum may play a part. Judging from my experiments, the estimation of the opsonic index should be made at the neutral reaction and in a diluted serum. The advisability of dilution has been pointed out especially by Simon and his co-workers; and with this idea the results of Neufeld and Rimpau with certain immune serums agree.

*The Influence of Acidity.*—Opsonins having been shown to be more active in a neutral than in an alkaline medium, the next point to be examined was the influence of the acid reaction upon the opsonization. Table V shows that opsonins are highly sensitive to the acid reaction.

TABLE V.

Neutralized Serum.	Amount of $\frac{1}{10}$ N. HCl Added to the Neutralized Serum.						Total Volume 5 c.c.
	0	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.5 c.c.	0.7 c.c.	
Dog	+++++	+++	++	+	±		Negative
Ox	++++++	+++	++	+	±		"
Pig	++++++	+++++	+++	+	±		"
Rabbit	++++	+++	++	+	±		"

If to the neutral serum is added 0.5 c.c. of  $\frac{1}{10}$  N. hydrochloric acid solution, no opsonic action is to be obtained. The quantity of acid indicated in the table was added to an amount of the neutralized serum corresponding to 1 c.c. of the original.

*Are Bacteria Opsonized in the Presence of Unfavorable Reaction?  
The restoration of opsonic activity.*

Bacteria suspended in a serum which contains enough acid or alkali to suppress opsonization under ordinary conditions were centrifugalized and washed in 0.9 per cent. salt solution. The washed bacteria were then mixed with leucocytes and incubated as usual. It was found that these bacteria were not taken up by leucocytes. Thus it can be concluded that when the reaction is unfavorable, opsonins do not attach themselves to the bacteria. An analogous phenomenon was encountered by Hektoen and Ruediger<sup>17</sup> in the case of certain antiopsonic neutral salts.

In these respects, opsonins seem to differ from most bacteriolytic and hæmolytic amboceptors, for they sensitize the corresponding cells in a medium containing an inhibitory amount of acid for opsonins. The sensitiveness of opsonins to slight degrees of acid and alkali is similar to complement, although opsonins surpass complements in sensitiveness.

The fact was ascertained that inactivation of opsonins by means of an unfavorable reaction is a reversible reaction. If the excess of acid or alkali is removed by neutralization the opsonins become active once more. In this respect they resemble complements.<sup>18</sup> A difference is observed in that opsonins are more active in neutral media, and complements more active in alkaline media. Further calcium chloride reduces the action of complements but not of opsonins, provided the concentration of this chemical used does not exceed  $\frac{1}{10}$  N.

Organic and inorganic acids in concentration of 1 N. render the serum opsonically inactive even after neutralization. The modification produced is irreversible.

Before leaving this topic, I wish to speak of an incident concerning the effect of small quantities of acids on the opsonic power of serum. At the beginning of this study, I employed  $\frac{1}{10}$  N. solution of various acids—hydrochloric, nitric, sulphuric, formic, acetic, propionic, lactic, butyric, oxybutyric, citric, tartaric, oxalic, male-

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<sup>17</sup> Hektoen and Ruediger, *Jour. of Infect. Diseases*, 1905, ii, 528.

<sup>18</sup> Noguchi, On the chemical inactivation and regeneration of complement. Read at the annual meeting of the Society of American Bacteriologists, Dec. 28, 1906.

inic, fumaric, itaconic, citraconic and glycerino-phosphoric—and as each in turn was added in small quantities to the serum the opsonic index was observed to rise. Contrary to my expectation, the addition of acid gradually but perceptibly increased the opsonic power of the serum. This first led me to think that acids might themselves act as opsonins. But the power was not further increased as the neutral point was reached, and from that point on, the increasing acidity finally suppressed it altogether.

#### EFFECT OF DESICCATION AND DRY HEAT ON OPSONINS.

*Effect of Desiccation.*—Opsonins are highly labile bodies. Their action disappears from the serum when it is allowed to stand for several days. In this respect they resemble complements. But no experiment has been recorded which tells us whether opsonins are obtainable in the dry state or not. I have tested this point. Normal serums of dog, ox and pig were dried at 23° C. with the aid of an air current, and the dissolved masses were tested again for their opsonic power. It was found that the opsonic power of the serum is not noticeably reduced by drying in this way. The next point was whether the opsonins would endure longer in the dried state. To determine this, I employed three samples of dried serum which had been preserved in the laboratory for two years. The serums were obtained from the rattlesnake, horse and ox, and were dried at a temperature approaching 48° C. Although I am unable to state the original activity at the time of drying, I can state that all three serums exhibited marked opsonic powers. Thus opsonins, once they are dried, are not labile substances.

On the other hand, dehydration of serum by means of alcohol renders the opsonins completely inactive. This fact would show that opsonins differ at least in this respect from many ferments, which stand treatment with alcohol, if not too prolonged.

*Effect of Dry Heat.*—The normal serum is robbed of its opsonic power by temperatures ranging from 55° to 60° C. Even immune serums, which are the most resistant in this respect, lost the greater part of their opsonic power at these temperatures, although traces may persist in a serum heated to 65° C. On the whole, opsonins are more thermostabile than complements, although less than the

usual immune bodies. The slight difference in the thermal resistance obtained by different investigators may depend upon differences in the reaction or some other physical or chemical conditions under which the tests were made.

Opsonins resemble ferments in their behavior to high temperature in the dry state. I have subjected the dried serums of ox, rabbit and dog to the temperature of 100°, 120°, 135° and 150° C., and then tested them for their opsonizing power. When the serums are heated to 120° C. and above, they become almost insoluble in water. To examine their activity, they must be powdered and emulsified in water. The bacterial emulsions were added to the former emulsions and the whole incubated at 37° C. for two hours. The result is given in Table VI.

TABLE VI.

	Opsonic Activity of the Heated or Unheated Dried Serum.					
	Unheated Dried Serum.	Dried Serum Heated to 100° c. c.	Dried Serum Heated to 120° C.	Dried Serum Heated to 135° C.	Dried Serum Heated to 150° C.	Unheated Dried Serum, Redissolved and then Heated to 56° C.
Ox serum	Active phagocytosis	Active phagocytosis	Active phagocytosis	Less active, but positive.	Very irregular occurrence of phagocytosis	Negative.
Rabbit serum (vaccinated)	"	"	"	"	"	"
Dog serum	"	"	"	"	"	"

From the foregoing experiment a high degree of resistance of the dried opsonins to high temperatures may be inferred. It is important to note that the dried serums regain their thermolability when redissolved in water, as is shown in the last column of the table.

Hence, it would appear that opsonins possess in common with ferments the property to resist in the dry state the action of high temperatures.

In the course of the experiments on desiccation and high temperature on opsonins, I took the opportunity of examining complements in the same manner.

It developed that complements do not disappear from the serum when it is dried at 23° C. The desiccated serum retained for several months complements unaltered in quantity and in activity.







Heating the dried serum to 100°, 120° and 135° C. does not deprive it of complementary action.

#### SUMMARY.

Opsonins reveal their maximum action in a medium of neutral reaction. No opsonization takes place in a serum which contains an amount of alkali corresponding to more than 1.6 c.c. of a  $\frac{1}{20}$  N. solution, or acid more than 0.5 c.c. of this concentration per 1 c.c. of serum. Of several normal blood serums titrated (lacmoid used as indicator) the average alkalinity was found to be equivalent to about 0.8 c.c. of  $\frac{1}{20}$  N. solution.

The opsonic index obtained in the native serums is not the expression of the action of the whole content of opsonins, but only so much as the degree of optimum of the reaction permits to come into action. Estimation of the opsonic power should, therefore, be made in a medium of neutral reaction and in diluted serum.

All serums have their opsonic power increased by diminishing the native alkalinity.

Opsonins whose activity is suspended by an unfavorable reaction become immediately active as soon as the reaction is brought back to the neutral point, unless the acid or alkali employed approaches the strength of 1 N., at which point the alteration becomes permanent.

Treatment of a serum with alcohol robs it of its opsonic power. The opsonic power of serum remains unaltered upon desiccation at 23° C. In the dry state opsonins are preserved for two years.

The temperatures of 100°, 120°, 135° and 150° C. do not destroy opsonins of the dried serum.

Complements of serum are also siccostabile and are preservable in that state for several months. Dry heat of 135° C. reduces but does not destroy the complementary power of the dried serum.

The opsonins and complements of the dried serum regain their original thermolability when they are dissolved in a proper amount of water.

Lastly, it may be emphasized that opsonins exhibit in their sensitiveness to reaction and resistance in the dry state to high temperatures certain properties characteristic of the ferments.



